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(54) ENCODED SELF-ASSEMBLING CHEMICAL LIBRARIES (ESACHEL)

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Description

Problem to be solved:

[0001] The isolation of specific binding molecules (e.g. organic molecules) is a central problem in chemistry, biology and pharmaceutical sciences. Typically, millions of molecules have to be screened, in order to find a suitable candidate. The preparation of very large libraries of organic molecules is typically cumbersome. Furthermore, the complexity associated with the identification of specific binding molecules from a pool of candidates grows with the size of the chemical library to be screened.

Solution:

[0002] In this invention, we use self-assembling libraries of organic molecules (typically forming dimers, trimmers or tetramers), in which the organic molecules are linked to an oligonucleotide which mediates the self-assembly of the library and/or provides a code associated to each binding moiety. The resulting library can be very large (as it originates by the combinatorial self-assembly of smaller sub-libraries). After the capture of the desired binding specificities on the target of interest, the "binding code" can be "decoded" by a number of experimental techniques (e.g., hybridization on DNA chips or by a modified polymerase chain reaction (PCR) technique followed by sequencing).

INTRODUCTION

[0003] The isolation of specific binding molecules (e.g., organic molecules) is a central problem in chemistry, biology and pharmaceutical sciences. For example, the vast majority of the drugs approved by the U.S. Food and Drug Administration are specific binders of biological targets which fall into one of the following categories: enzymes, receptors or ion channels. The specific binding to the biological target is not *per se* sufficient to turn a binding molecule into a drug, as it is widely recognized that other molecular properties (such as pharmacokinetic behaviour and stability) contribute to the performance of a drug. However, the isolation of specific binders against a relevant biological target typically represents the starting point in the process which leads to a new drug [Drews J. Drug discovery: a historical perspective. Science (2000) 287:1960-1964].

[0004] The ability to rapidly generate specific binders against the biological targets of interest would be invaluable also for a variety of chemical and biological applications. For example, the specific neutralization of a particular epitope of the intracellular protein of choice may provide information on the functional role of this epitope (and consequently of this protein). In principle, the use of monoclonal antibodies specific for a given epitope may provide the same type of information [Winter G, Griffiths AD, Hawkins RE, Hoogenboom HR. Making antibodies by phage display technology. Annu Rev Immunol. (1994) 12:433-455]. However, most antibodies do not readily cross the cell membrane and have to be artificially introduced into the cell of interest. In principle, intracellular antibodies can also be expressed into target cells by targeted gene delivery (e.g., by cell transfection with DNA directing the expression of the antibody). In this case, the antibody often does not fold, as the reducing intracellular milieu does not allow the formation of disulfide bonds which often contribute in an essential manner to antibody stability [Deslderio A, Franconi R, Lopez M, Villani ME, Viti F, Chiaraluce R, Consalvi V, Neri D, Benvenuto E. A semi-synthetic repertoire of intrinsically stable antibody fragments derived from a single-framework scaffold. J Mol Biol. (2001) 310: 603-615]. High affinity binding molecules amenable to chemical synthesis may provide a valuable alternative to antibody technology.

[0005] In Chemistry and Materials Sciences, the facile isolation of specific binding molecules may be useful for purposes as diverse as the generation of biosensors, the acceleration of chemical reactions, the design of materials with novel properties, the selective capture/separation/immobilization of target molecules.

[0006] The generation of large repertoires of molecules (e.g., by combinatorial chemistry; Otto S, Furlan RL, Sanders JK. Dynamic combinatorial chemistry. Drug Discov Today. (2002) 7: 117-125), coupled to ingenious screening methodologies, is recognized as an important avenue for the isolation of desired binding specificities. For example, most large pharmaceutical companies have proprietary chemical libraries, which they search for the identification of lead compounds. These libraries may be as large as > 1 million members and yet, in some instances, not yield the binding specificities of interest [Böhm HJ, Stahl M. Structure-based library design: molecular modeling merges with combinatorial chemistry. Current Opinion in Chemical Biology (2000) 4: 283-286]. The screening of libraries containing millions of compounds may require not only very sophisticated synthetic methods, but also complex robotics and infrastructure for the storage, screening and evaluation of the members of the library.

[0007] The generation of large macromolecular repertoires (e.g., peptide or protein libraries), together with efficient biological and/or biochemical methods for the identification of binding specificities (such as phage display [Winter, 1994], peptides on plasmids [Cull MG, Miller JF, Schatz PJ. Screening for receptor ligands using large libraries of peptides linked to the C terminus of the lac repressor. Proc Natl Acad Sci U S A. (1992) 89: 1865-1869] ribosome display [Schaffitzel

C, Hanes J, Jermutus L, Pluckthun A. Ribosome display: an in vitro method for selection and evolution of antibodies from libraries. J Immunol Methods. (1999) 231: 119-135] yeast display [Boder ET, Wittrup KD. Yeast surface display for screening combinatorial polypeptide libraries. Nat Biotechnol. (1997) 15: 553-557], periplasmic expression with cytometric screening [Chen G, Hayhurst A, Thomas JG, Harvey BR, Iverson BL, Georgiou G. Isolation of high-affinity ligand-binding proteins by periplasmic expression with cytometric screening (PECS). Nat Biotechnol. (2001) 19:537-542], iterative colony filter screening [Giovannoni L, Viti F, Zardi L, Neri D. Isolation of anti-anglogenesis antibodies from a large combinatorial repertoire by colony filter screening. Nucleic Acids Res. (2001) 29: E27] etc.) may allow the isolation of valuable polypeptide binders, such as specific monoclonal antibodies, improved hormones and novel DNA-binding proteins. In contrast to conventional chemical libraries, protein libraries in the embodiments mentioned above may allow the efficient screening of as many as 1-10 billion individual members, in the search of a binding specificity of interest. On one hand, the generation of libraries of genes (e.g., the combinatorial mutagenesis of antibody genes; Winter, 1994; Viti F, Nilsson F, Demartis S, Huber A, Neri D. Design and use of phage display libraries for the selection of antibodies and enzymes. Methods Enzymol. (2000) 326:480-505) can directly be translated into libraries of proteins, using suitable expression systems (e.g. bacteria, yeasts, mammalian cells). Furthermore, methods such as phage display produce particles in which a *phenotype* (typically the binding properties of a protein, displayed on the surface of filamentous phage) is physically coupled to the corresponding *genotype* (i.e., the gene coding for the protein displayed on phage) [Winter, 1994], allowing the facile amplification and identification of library binding members with the desired binding specificity.

[0008] However, while biological/biochemical methods for the isolation of specific binding biomacromolecules can provide very useful binding specificities, their scope is essentially limited to repertoires of polypeptides or of nucleic acids [Brody EN, Gold L. Aptamers as therapeutic and diagnostic agents. J Biotechnol. (2000) 74:5-13]. For some applications, large biomacromolecules (such as proteins or DNA) are not ideal. For example, they are often unable to efficiently cross the cell membrane, and may undergo hydrolytic degradation *in vivo*.

[0009] In an attempt to mimic biological/biochemical methods for the identification of organic molecules with desired binding properties, out of a chemical library, Brenner and Lerner [Brenner S, Lerner RA. Encoded combinatorial chemistry. Proc Natl Acad Sci U S A. (1992) 89: 5381-5383] have proposed the use of encoded chemical libraries (ECL). In their invention, the authors conceived a process of alternating parallel combinatorial synthesis in order to encode individual members of a large library of chemicals with unique nucleotide sequences. In particular, the authors postulated the combinatorial synthesis of polymeric chemical compounds on a solid support (e.g., a bead), where a step in the combinatorial synthesis would be followed by the synthesis (on the same bead) of a DNA sequence, to be used as a "memory tag" for the chemical reactions performed on the bead. In typical applications, DNA-encoded beads would be incubated with a target molecule (e.g., a protein of pharmaceutical relevance). After the DNA-tagged bead bearing the polymeric chemical entity is bound to the target, it should be possible to amplify the genetic tag by replication and use it for enrichment of the bound molecules by serial hybridization to a subset of the library. The nature of the polymeric chemical structure bound to the receptor could be decoded by sequencing the nucleotide tag.

[0010] The ECL method has the advantage of introducing the concept of "coding" a particular polymeric chemical moiety, synthesized on a bead, with a corresponding oligonucleotidic sequence, which can be "read" and amplified by PCR. However, the ECL method has a number of drawbacks. First, a general chemistry is needed which allows the alternating synthesis of polymeric organic molecules (often with different reactivity properties) and DNA synthesis on a bead. Second, the synthesis, management and quality control of large libraries (e.g., > 1 million individual members) remains a formidable task. In fact, the usefulness of the ECL method has yet to be demonstrated with experimental examples.

[0011] From US 5,573,905 an encoded combinatorial chemical library is known which comprises a plurality of bifunctional molecules according to the formula A-B-C, where A is a polymeric chemical moiety. B is a linker molecule operatively linking A and C, consisting of a chain length of 1 to about 20 atoms and preferably comprising means for attachment to a solid support. C is an identifier oligonucleotide comprising a sequence of nucleotides that identifies the structure of the chemical moiety. The attachment to a solid support is especially preferred when synthesizing step by step the chemical moiety (a polymer built of subunits X_{1-n}) and the oligonucleotide (built of nucleotides Z_{1-n} which code for and identify the structure of the chemical subunits of the polymer). Also described are the bifunctional molecules of the library, and methods of using the library to identify chemical structures within the library that bind to biological active molecules in preselected binding interactions. Utilizing the code C for the identification of the polymer A and attaching the code C to the polymer A with a linker molecule B allows the polymer to be identified exactly, however, the solution presented in US 5,573,905 (which basically is the same as published by Brenner and Leer, 1992) is limited to this special type of a chemical moiety. The fact that Individual synthesis has to be carried out for each individual of a chemical library is regarded as another disadvantage.

[0012] Dynamic combinatorial chemistry has established itself as one avenue for the reversible (covalent or non-covalent) assembly of binding moieties, some of which may be stabilized by the presence of a target molecule (e.g., a target protein), thus allowing the recovery of the stabilized complex [Ramström and Lehn (2002) "Drug discovery by

dynamic combinatorial libraries". Nature Reviews Drug Discovey, Vol. 1, page 26-36].

[0013] DE 196 19 373 describes a strategy for the implementation of dynamic combinatorial chemical libraries, relying on the reversible assembly of oligonucleotide strands (or analogues), each strand being chemically modified with a polymeric or monomeric chemical entity and being capable of forming heteroduplexes. Assembly and disassembly of chemically-modified oligonucleotides is reported to be essential until a target molecule (e.g., a target protein or "substrate") stabilizes certain supramolecular assemblies. In a typical implementation of the techno logy, a long (chemically modified) oligonucleotide is used to direct the transient self-assembly with shorter (chemically modified) oligonucleotides forming reversible heteroduplexes with distinctive portions of the long oilgonucleotide. Typically, the same oligonucleotide sequences are used for different chemical compounds, as this ensures the equivalent re-shuffling propensities of the different library members. However, the technology disclosed presents the same problems as most dynamic combinatorial chemical libraries, i.e. the difficult identification of binding complexes (particularly when large libraries are used). WO 00/23458 discloses a modification of conventional split and pool combinatorial chemistry techniques (normally limited to compounds on beads). The carrier for the synthesis of a polymeric (or at least modular) chemical compound is a long DNA strand which is characterized by the simultaneous presence of a site for the growth of the polymeric chemical compound and specific oligonucleotide sequences (separated by linking regions) that permit the specific and orthogonal purification of synthesis intermediates on resins carrying a complementary oligonucleotide sequence. The resulting different synthesis intermediates, carrying different oligonucleotide codes, can be chemically modified with a monomer at a specific reaction site and then pooled. The cycle may be repeated, thus allowing the growth of an encoded polymeric (or at least modular) chemical structure linked to DNA. This synthetic structure lends itself to "DNA-shuffling" and re-synthesis for directed evolution selective strategies. Its inherent design limits this strategy to polymeric (or at least modular) chemical compounds, whose synthesis does not interfere with the simultaneous presence of an oligonucleotide. The same limit applies to each compound in the library requiring either the pre-synthesis or the growing synthesis of individual DNA molecules (i.e., 10^6 DNA molecules for 10^6 chemical compounds). Due to the length of the DNA oligonucleotide that supports the chemical structure problems in its synthesis and during the selection with target molecules are to be expected. DE 197 41 716 discloses a recognition system consisting of at least one immobilized binding partner A (capable of binding to at least B) and at least one binding partner B (capable of binding to binding partner A). Tagging of individual molecules with individual oligonucleotide tags (similar to what was previously described in US 5,573,905 or published [Sano T, Smith CL, Cantor CR. (1992) Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates. Science, vol. 258, pages 120-122]) is disclosed, in order to facilitate the identification of enriched tagged molecules by means of electrochemical devices, carrying "reader"-complemantary oligonucleotides. For example, the tagging of individual antibodies with distinctive oligonucleotides is proposed as a method for the detection of a selective binding reaction.

[0014] US 5,573,905 describes two alternating parallel combinatorial syntheses, such that a genetic tag is chemically linked to a polymeric chemical structure being synthesized. In this method, the addition (typically on a bead) of a chemical unit (e.g., an aminoacid) is followed by the addition of an oligonucleotide sequence, which functions as an identifier for the structure of the polymeric compound. A library is built up by the repeating process after pooling and division of the reaction products obtained at each step. Limitations of this method comprise that the size of the library which can be synthesized and used is restricted to the number of beads required for the synthesis, at least one bead-compound being necessary for a successful selection. "Encoding" of polymeric chemical structures only applies via alternating chemical syntheses.

[0015] It is therefore an object of the present Invention to provide a chemical compound comprising a chemical moiety of any kind capable of performing a binding interaction with a target molecule (e.g. a biological target) and further comprising an oligonucleotide or functional analogue thereof which chemical compound does not need to be individually synthesized in order to build up a chemical library.

[0016] This object is met according to a first aspect by the combination of features of independent claim 1, defining a chemical library comprising combination reaction products of at least two chemical compounds, each one of these chemical compounds comprising:

a) a chemical moiety (p,q) potentially capable of performing a binding interaction with a single target molecule;
b) an oligonucleotide (b,b'), a part of which being a self-assembly moiety (m,m');

the chemical compounds being bound to each other by the self-assembly moleties (m,m') of their oligonucleotides (b, b'). The chemical library according to the Invention is characterized in that the combination reaction product is stable In the absence of said target molecule, wherein the oligonucleotides (b,b') of each one of the chemical compounds comprise a variable, unique coding sequence (b2,b2') individually coding for the identification of the particular chemical moiety (p,q).

[0017] Further aspects and features of the present invention derive from the dependent claims.

SUMMARY OF THE INVENTION

[0018] In our invention, we reasoned that a key contribution to the generation (and screening) of very large chemical libraries may come from the "self-assembly" of encoded molecules. In particular, we reasoned that self-assembly (e.g., by homodimerization, heterodimerization or multimerization) of DNA-tagged chemical entities would represent an avenue for the facile generation of very large DNA-tagged chemical libraries, starting from smaller DNA-tagged chemical libraries. For example, self-assembly (heterodimerization) of two libraries containing 1000 members would yield 1'000'000 different combinations, i.e. 1'000'000 different chemical entities. Notably, homo- or hetero-trimerization of encoded libraries containing 1000 DNA-tagged members would yield a library containing 1'000'000'000 different DNA-tagged combinations, i.e. chemical entities. Thus, the present invention provides a chemical library comprising combination reaction products of at least two chemical compounds comprising a chemical moiety of any kind capable of performing a binding Interaction with a target molecule (e.g. a biological target) and further comprising an oligonucleotide or functional analogue thereof which can be synthesized separately and then coupled together. The resulting chemical derivative(s) of the oligonucleotide can further assemble with other similar compounds to generate higher order structures and encoded libraries of compounds.

[0019] For illustrative purposes, one particular embodiment of our invention is depicted in Figure 1. Two chemical sub-libraries are synthesized by chemical modification of the 3' end and the 5' end, respectively, of oligonucleotides capable of duplex formation and which carry distinctive "sequence tags" (associated with [and therefore "coding for"] the chemical moiety attached to their extremity). The resulting encoded self-assembled chemical library (ESACHEL) can be very large (as it originates from the combinatorial self-assembly of two smaller libraries) and can be screened for binding to a biological target (e.g., a protein of pharmaceutical interest). Those members of the library which display suitable binding specificities can be captured with the target of interest (for example, using a target immobilized on a solid support). Their genetic code, encoding the chemical entity responsible for the binding specificity of interest, can then be retrieved using a number of ingenious methods, which are described In the section "Description of the Invention" (see below).

DEFINITIONS

Specific:

[0020] This may be used to refer to the situation in which one member of a specific binding pair will not show any significant binding to molecules other than its specific binding partner(s). In general, specificity is associated with a significant difference in binding affinity, relative to "non-specific" targets. The term is also apPlicable where e.g. a binding member is specific for a particular surface on the target molecule (hereafter termed as " epitope"), in which case the specific binding member with this specificity will be able to bind to various target molecules carrying the epitope.

FIGURE CAPTIONS

Figure 1: A simple embodiment of ESACHEL technology:

[0021] In a simple embodiment of ESACHEL technology, two chemical sub-libraries are synthesized by individual chemical modification of the 3' end and the 5' end, respectively, of oligonucleotides capable of partial heteroduplex formation and which carry distinctive "sequence tags" (associated with [and therefore "coding for"] the chemical moieties **p** and **q** attached to their extremity). The resulting encoded self-assembled chemical library (ESACHEL) can be very large (as it originates from the combinatorial self-assembly of two smaller libraries) and can be screened for binding to a biological target (e.g., a protein of pharmaceutical interest). Those members of the library which display suitable binding specificities can be captured with the target of interest (for example, using a target immobilized on a solid support). Their genetic code, encoding the chemical entity responsible for the binding specificity of interest, can then be retrieved using a number of ingenious methods

Figure 2: Generalization of the ESACHEL design:

[0022] The main ingredients of ESACHEL technology are **chemical compounds**, comprising an **oligonucleotidic moiety** (typically, a DNA sequence) linked to an **oligomerization domain** [capable of mediating the (homo- or hetero-) dimerization, trimerization or tetramerization of the **chemical compounds**], linked to a **chemical entity**, which may be involved in a specific binding interaction with a target molecule. Part of the sequence of the oligonucleotidic moiety will be uniquely associated with the chemical entity (therefore acting as a "code"). The oilgomerization domain and the code can be distinct portions of the same molecule (typically an oligonucleotide).

Figure 3: Self-assembly of Individual ESACHEL chemical compounds can yield combinatorial libraries of large size:

[0023] In a practical embodiment of ESACHEL technology, a number n of different chemical compounds, carrying a thiol-reactive moiety (e.g., a maleimido or a Iodoacetamido group), are reacted (in separate reactions) with n different DNA oligonucleotides, carrying a thiol group at the 3' end. The corresponding pool of n conjugates is Indicated in the Figure as "pool A". Similarly, a number m of different chemical compounds, carrying a thiol-reactive moiety (e.g., a maleimido or a iodoacetamido group), are reacted (in separate reactions) with **m** different DNA oligonucleotides, carrying a thiol group at the 5' end. The corresponding pool of **m** conjugates is indicated in the Figure as "pool B". The resulting self-assembled library members will correspond to **m** × **n** combinations.

Figure 4: Large library sizes can be achieved by ESACHEL embodiments, in which trimerization domains or tetramerization domains are DNA sequences forming triplexes or quadruplexes:

[0024] Certain DNA sequences are known to be capable of forming stable trimeric complexes or stable tetrameric complexes. For example, Hoogsten pairing of DNA triplexes could allow the self-assembly of Pools A × B × C, containing **n, m** and **l** members, respectively. The tetrameric assembly of DNA-(chemical moiety) conjugates would allow even larger library sizes, starting from sub-libraries A, B, C and D of small dimension.

Figure 5: One method of ESACHEL decoding:

[0025] The oligonucleotides of sub-library A bear chemical entities at the 3' end. Towards the 3' extremity, the DNA sequence is designed to hybridize to the DNA sequences at the 5' extremity of oligonucleotides of sub-library B. The hybridization region is interrupted by a small segment. In sub-library A, this small segment is conveniently composed of phosphodiester backbone without bases (termed d-spacer in the Figure); In sub-library B, the corresponding short segment will have unique sequence for each member of the sub-library (therefore acting as "code" for the sub-library B). By contrast, oligonucleotides of sub-library A have their distinctive code towards the 5' extremity.

[0026] After biopanning, oligonucleotides of sub-library B remain stably annealed to oligonucleotides of sub-library A, and can work as primers for a DNA polymerase reaction on the template A. The resulting DNA segment, carrying both code A and code B, can be amplified (typically by PCR), using primers which hybridize at the constant extremities of the DNA segment.

Figure 6: A general method of ESACHEL decoding:

[0027] The identity of specific binders, isolated from sub-libraries A and B carrying chemical moieties at the extremities of partially-annealing oligonucleotides, is established by hybridization with target oligonucleotides immobilized on one or more chips. Such chips preferably are made from silicon wafers with attached oligonucleotide fragments. For example, chip A will allow the reading of the identity (and frequency) of members of sub-library A, rescued after a biopanning experiment. Similarly, chip B will allow the reading of the identity (and frequency) of members of sub-library B. In a first step, the decoding method depicted in the Figure will not provide information about the pairing of code A and code B within specific binding members. However, decoding on chip A and B will suggest candidate components of sub-libraries A and B, to be re-annealed and screened in a successive round of bio-panning. Increasingly stringent binding to the target will be mirrored by a reduction in the number of A and B members, identified on the chip. Ultimately, the possible combinations of the candidate A and B members will be assembled Individually (or in smaller pools), and assayed for binding to the target.

Figure 7: A PCR-based method for ESACHEL deconvolution:

[0028] Sub-libraries A and B form a heteroduplex, flanked by unique sequences coding for the different library members and by constant DNA segments at the termini. After biopanning, suitable pairs of primers allow the PCR amplification of the two strands, yielding PCR products whose sequence can be identified using standard methods (e.g. by concatenation of the PCR products, followed by subcloning and sequencing. A deconvolution procedure may be applied (consisting of one or more rounds of panning, followed by sequencing and by the choice of a restricted set of sub-library components for the next ESACHEL screening), restricting the number of candidate ESACHEL members capable of giving specific binders after self-assembly.

Figure 8: Converting ESACHEL ligands into covalently-linked chemical moieties:

[0029] In many ESACHEL embodiments, chemical derivatives of self-assembling oligonucleotides will be isolated at the end of one or more rounds of panning. For many applications, it will be desirable to covalently link together the chemical moieties, responsible for the interaction with the target molecule of interest. The length, rigidity, stereoelectronic chemical properties and solubility of the linker will influence the binding affinity and performance of the resulting molecule.

Figure 9: Chemical equilibria contributing to the chelate effect:

[0030] The diagram shows the possible states of the interactions between a bidentate ligand (A-B) binding to a target molecule. In state nI, both A and B moieties are bound to their respective binding pockets. In state nII and nIII only moiety A or B are bound, respectively. In state nIV, the compound A-B is dissociated from the target.

[0031] A computer program has been written for the approximate evaluation of the contribution of the chelate effect to the residence time of A-B on the target in irreversible dissociation conditions, as a function of kinetic association and dissociation constants of the moieties A and B towards their respective binding pockets, and of the linker length between A and B. The probability that one moiety dissociates per time unit is indicated as poff. The probability that one moiety binds to the target per time unit is indicated as pon.

[0032] **Figure 10: Assembly of molecule p with molecular repertoire Q:** The diagram shows heteroduplex formation between an oligonucleotide, coupled to a low-affinity binder **p**, and a second class of oligonucleotides, which bear chemical moieties **q** and distinctive codes, capable of identifying the molecules **q** which synergise with **p** for binding to a target molecule (e.g., a protein target).

DESCRIPTION OF THE INVENTION

[0033] The main ingredients of ESACHEL technology **are chemical compounds**, comprising an **oligonucleotidic moiety** (typically, a DNA sequence) linked to **an oligomerization domain** [capable of mediating the (homo- or hetero-) dimerization, trimerization or tetramerization of **the chemical compounds],** linked to **a chemical entity**, which may be involved in a specific binding interaction with a target molecule (Figure 2). Part of the sequence of the oligonucleotidic moiety will be uniquely associated with the chemical entity (therefore acting as a "code"). In many cases, portions of the same oligonucleotide will serve as the oligomerization domain and the code.

Oligonucleotidic moiety:

[0034] The nature and the design of the oligonucleotidic moieties In ESACHEL technology is best understood by the description of the "coding" system, provided in the sections below and In the Examples. As an introduction, it suffices to say that, by stably associating chemical entities with a unique oligonucleotidic sequence (e.g., a sequence of DNA or DNA analogues), one provides that chemical entity with a unique code, which can be "read" in a variety of ways (sequencing, hybridization to DNA chips, etc.) and which may be amenable to amplification (e.g., by the use of the polymerase chain reaction [PCR]). Furthermore, using ingenuous methods described below, the code of one particular **chemical compound** may become physically linked to the code of other **chemical compound(s)**, when these **chemical compounds** are associated by means of an **oligomerization domain.**

Oligomerization domains:

[0035] Suitable DNA sequences (capable of heteroduplex, triplex [Strobel SA, Dervan PB. Single-site enzymatic cleavage of yeast genomic DNA mediated by triple helix formation. Nature. (1991) 350:172-174] or quadruplex [Various authors. Issue of Biopolymers (2000-2001), volume 56 (3)] formation), can be considered as possible oligomerization domains.

[0036] Alternatively, but not as a part of the present invention, the use of other self-assembling polypeptides (e.g. amphipathic peptide helices such as leucine zippers) could be considered. Many more chemical moieties could be considered as mediators of chemically defined, oligomeric moieties. For instance, complexes of metal atoms with suitable ligands [such as dipyridyl or tripyridyl derivatives) could be envisaged. Furthermore, one could envisage that a non-covalent Interaction would bring together different chemical compounds, which could then react with one another and become covalently associated.

Some practical embodiments of ESACHEL technology:

[0037] In order to illustrate one possible practical embodiment of ESACHEL technology, let us consider the following

example (depicted in Figure 3).

[0038] A number **n** of different chemical compounds, carrying a reactive moiety (e.g. a thiol-reactive maleimido or a iodoacetamido group), are reacted (in separate reactions) with n different DNA oligonucleotides, carrying a reactive moiety (e.g. a thiol group at the 3' end). The corresponding pool of n conjugates is indicated in the Figure as "pool A". The oligonucleotides of pool A are designed to have:

- one portion of the DNA sequence which can hybridize to compounds of pool B (see Figure 3 and comments below)
- a distinctive DNA sequence for each of the **n** members of Pool A
- additional portions of the DNA sequence judiciously designed for "decoding" binding combinations (optional; see below in the section about "Decoding" and in the Examples).

[0039] Similarly, a number **m** of different chemical compounds, carrying a thiol-reactive moiety (e.g., a maleimido or a iodoacetamido group), are reacted (in separate reactions) with m different DNA oligonucleotides, carrying a thiol group at the 5' end. The corresponding pool of m conjugates is indicated in the Figure as "pool B".

[0040] The oligonucleotides of pool B are designed to have:

- one portion of the DNA sequence which can hybridize to compounds of pool A (see Figure 3)
- a distinctive DNA sequence for each of the m members of Pool B
- additional portions of the DNA sequence judiciously designed for "decoding" binding combinations (optional).

[0041] The partially complementary strands of the DNA conjugates of pool A and pool B can easily heterodimerize in solution, with comparable efficiency within the different n members of Pool A and the m members of Pool B. If suitable stoichiometric ratios of the compounds of Pool A and Pool B are used, the n different types of compounds of Pool A will heterodimerize with the m different types of compounds of Pool B, yielding a combinatorial self-assembled chemical library of dimension **m** $\times$ **n.** For example, two libraries of thousands of compounds would yield millions of different combinations. Furthermore, the resulting self-assembled **m** $\times$ **n** combinations will carry unique DNA codes, corresponding to the non-covalent but stable association (heterodimerization) of the DNA code of the member of Pool A with the DNA code of the member of Pool B.

[0042] As an alternative to the coupling of chemical entities to thiol-bearing oligonucleotides, a number of standard chemical alternatives can be considered (e.g., oligonucleotides carrying a phosphodiester bond at one extremity, forming chemical structures such as -O-P(O)$_2$-O-(CH$_2$)$_n$-NH-CO-R, where R may correspond to a number of different chemical entities, and n may range between 1 and 10).

[0043] Let us assume that one particular member of the library is capable of specific binding to a target of interest (e.g., a protein immobilized on a bead). Let us also assume that both strands A and B contributed to the specific binding interaction (for a discussion of the chelate effect which may facilitate this specific binding, see below). It should be possible to preferentially enrich this particular combination of A and B over the **m** $\times$ **n** combinations (for example, by exposing the library to the target protein on the bead, followed by the physical removal of the bead from the library solution, followed by judicious washing of the bead, to reduce the amount of non-specific binders on the bead). The analogy of this procedure to the biopanning procedures used with antibody phage libraries (Viti, 2000) should be evident to any person skilled in the art.

[0044] The rescue of the particular combination of A and B, displaying the desired binding specificity, can then be followed by the identification of the chemical entities responsible for the binding, by identifying the DNA codes of the two strands A and B [see later section on "decoding" for a discussion on possible strategies for the identification of the DNA codes].

[0045] For a number of applications, at the end of the ESACHEL procedure, it may be desirable to link the two chemical entities A and B, responsible for the specific binding to the target (Figure 3). One may want to try a variety of different chemical linkers, and assess whether the resulting chemical compounds, derived from the chemical entities A and B, display desired molecular properties (e.g., high affinity for the target, high specificity for the target, suitable chemical stability, suitable solubility properties, suitable pharmacological properties, etc.). For example, the length of the chemical linker between A and B will dramatically influence he binding properties of the conjugate (for a discussion, see section below on the chelate effect).

[0046] In the case of Figure 3, a DNA portion is used as heterodimerization domain, and thioether bond formation is used for the coupling of DNA oligonucleotides to chemical entities of the library. However, other oligomerization domains could be considered, as well as other chemical avenues for the coupling of chemical entities to DNA.

[0047] Certain DNA sequences are known to be capable of forming stable trimeric complexes [Strobel, 1991] or stable tetrameric complexes [Various authors, 2000-2001]. For example, Hoogsten pairing of DNA triplexes could allow the self-assembly of Pools A $\times$ B $\times$ C, containing n, m and l members, respectively (Figure 4). The tetrameric assembly of DNA-(chemical moiety) conjugates would allow even larger library sizes, starting from sub-libraries A, B, C and D of

small dimension. However, the decoding of binding interactions can, in some cases, be more difficult for trimeric and/or tetrameric self-assembled encoded libraries, as compared to dimeric libraries. Furthermore, the length and flexibility of the linkers between the DNA strands and the chemical moieties displayed at their extremity may either facilitate or hinder the identification of specific binding members of the encoded self-assembled chemical (ESACHEL) library. A certain degree of flexibility may allow suitable chemical moieties to find complementary pockets on the target molecule (Figure 4). On the other hands, the affinity contribution of the chelate effect is expected to decrease with linker length.

[0048] It is worth mentioning that, starting from sub-libraries of 100 members, trimeric ESACHEL libraries would contain 10^6 members, while tetrameric ESACHEL libraries would contain 10^8 members. It is easy to calculate the resulting library size, starting from sub-libraries of different dimension. The large combinatorial complexity of encoded self-assembling chemical compounds may allow the identification of specific binding members, which have so far escaped identification using conventional combinatorial chemical methods. An analogy can be drawn from the field of antibody phage technology, where it was demonstrated that library size plays a crucial role in the isolation of high-affinity antibodies.

ESACHEL codes and decoding methods:

[0049] In ESACHEL technology, unique oligonucleotidic sequences (typically, DNA sequences) provide chemical entities with a unique code. How many different sequences do we need, in order to identify members of a library?

[0050] As mentioned above, the key components of ESACHEL technology are **chemical compounds**, comprising an **oligonucleotidic moiety** (typically, a DNA sequence) linked to **an oligomerization domain**, in turn linked to a **chemical entity.** In most cases, the oligonucleotidic moiety will also provide the oligomerization domains. As a consequence, in most cases, ESACHEL components will be chemical entities coupled to judiciously chosen DNA oligonucleotides. Typically, such oligonucleotides will contain a constant part and a variable part (uniquely characteristic for each member of the library).

[0051] Let us consider, as an example, the case illustrated in Figure 3, and discussed in the section "Some practical embodiments of ESACHEL technology, (see above). In this example, a sub-library "A" (containing n compounds attached at the 3' extremity of DNA oligonucleotides) is assembled to a sub-library "B" (containing m compounds attached at the 5' extremity of oligonucleotides). Sub-library A can be represented by a DNA sequence of $\times$ bases, where 4^x is greater or equal to n. Sub-library B can be represented by a DNA sequence of y bases, where 4^y is greater or equal to m. In most cases (see below), identification of the code of sub-library members also provides information about which sub-library a particular code (and therefore a particular compound) belongs to.

[0052] Many methods of "decoding" the ESACHEL codes could be envisaged. Below, we illustrate three, which correspond to different experimental requirements and which demonstrate the flexibility of ESACHEL technology.

[0053] Let us consider for simplicity the ESACHEL embodiment of Figure 3. A convenient design of oligonucleotides, on which sub-libraries A and B are based, is depicted in Figure 5. The oligonucleotides of sub-library A bear chemical entities at the 3' end. Towards the 3' extremity, the DNA sequence is designed to hybridize to the DNA sequences at the 5' extremity of oligonucleotides of sub-library B. The hybridization region is interrupted by a small segment. In sub-library A, this small segment is conveniently composed of phosphodiester backbone without bases (termed d-spacer in the Figure); in sub-library B, the corresponding short segment will have unique sequence for each member of the sub-library (therefore acting as "code" for the sub-library B). By contrast, oligonucleotides of sub-library A have their distinctive code towards the 5' extremity.

[0054] After biopanning, it is desirable to learn about the code of the binding members displaying a desired binding specificity. Oligonucleotides of sub-library B remain stably annealed to oligonucleotides of sub-library A, and can work as primers for a DNA polymerase reaction on the template A. The resulting DNA segment, carrying both code A and code B, can be amplified (typically by PCR), using primers which hybridize at the constant extremities of the DNA segment (Figure 5).

[0055] If several specific binding members are isolated at the end of a biopanning experiment, several PCR products will be generated with the process illustrated in Figure 5. These products will have similar sequences, except for the regions coding for A and B sub-library members. In order to learn more about the relative abundance of the different specific binding members, it will be convenient to create concatenamers, starting from the various PCR products in the reaction mixture. Such concatenated sequences can be "read" by sequencing, revealing both the identity and the frequency of pairs of code A and code B (which uniquely correspond to particular library members).

[0056] An alternative decoding strategy is depicted in Figure 6. Sub-libraries A and B carry chemical moieties at the extremities of partially-annealing oligonucleotides. In most cases, the DNA portion forming a heteroduplex will be constant within the library. Conversely, the other extremities will be designed in a way that heteroduplex formation is disfavored. Such unpaired DNA strands will be available for hybridization with target oligonucleotides (for example, DNA oligonucleotides immobilized on one or more chips). For example, chip A will allow the reading of the identity (and frequency) of members of sub-library A, rescued after a biopanning experiment. Similarly, chip B will allow the reading of the identity (and frequency) of members of sub-library B. A variety of strategies can be considered (e.g., DNA radiolabeling, DNA

blotinylation followed by detection with streptavidin-based reagents, etc.) for the visualization of the binding reaction on the chip.

[0057] In a first step, the decoding method of Figure 6 will not provide information about the pairing of code A and code B within specific binding members. However, decoding on chip A and B will suggest candidate components of sub-libraries A and B, to be re-annealed and screened in a successive round of bio-panning. Increasingly stringent binding to the target will be mirrored by a continuous reduction in the number of A and B members, identified on the chip. Ultimately, the possible combinations of the candidate A and B members will be assembled individually (or in smaller pools), and assayed for binding to the target. We refer to this iterative strategy as **deconvolution.**

[0058] Obviously, the decoding method of Figure 6 is valid also for ESACHEL, when libraries self-assemble to form trimeric or tetrameric complexes (e.g. using DNA triplexes or quadruplexes for the oligomerization of compounds). In these cases, 3 or 4 chips may be used, respectively, which carry distinctive target oligonucleotides for decoding.

[0059] If appropriate, the DNA of selected binding moieties of Figure 6 may be PCR amplified prior to chip hybridization. In this case, oligonucleotide design will resemble the one described in the next paragraph (see also Figure 7).

[0060] Another possible decoding method is illustrated in Figure 7. Sub-libraries A and B form a heteroduplex, flanked by unique sequences coding for the different library members and by constant DNA segments at the termini. After biopanning, suitable pairs of primers allow the PCR amplification of the two strands, yielding PCR products whose sequence can be identified using standard methods (e.g. by concatenation of the PCR products, followed by subcloning and sequencing. Similar to the chip-based method illustrated in Figure 6, the method of Figure 7 will not provide, In general, direct information about pairing of code A and code B in specific binding members. However, (similar to what described for Figure 6), a deconvolution procedure may be applied (consisting of one or more rounds of panning, followed by sequencing and by the choice of a restricted set of sub-library components for the next ESACHEL screening), restricting the number of candidate ESACHEL members capable of giving specific binders after self-assembly.

Library construction:

[0061] ESACHEL library construction is facilitated not only by the large dimension that can be achieved by self-assembly of sub-libraries, but also by the facile generation and purification of chemical derivatives of DNA oligonucleotides.

[0062] As mentioned above, DNA oligonucleotides, bearing a thiol group at their 3' or 5' end, can be purchased from a variety of commercial suppliers. The chemistry of the modification of thiol groups with reagents bearing reactive groups such as iodoacetamido moieties or maleimido moieties is well-established [see for example the molecular Probes catalogue at www.probes.com]. Furthermore, several methods are available in the literature for the chemical modification of 3' or 5' extremities of DNA oligonucleotides, for example during solid phase synthesis procedures.

[0063] Chemical derivatives of DNA (or some DNA analogues) have the characteristic property of being highly negatively charged at neutral pH. This facilitates the development of general purification strategies of the DNA derivatives. For example, anion exchange chromatography allows the non-covalent (but stable) immobilization of DNA oligonucleotides (and their derivatives) on a resin, while other components of a reaction mixtures can be washed away. DNA derivatives can then be eluted by buffer change. Alternatively, other purification methods (e.g. reverse phase chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography etc.) could be considered.

[0064] The availability of generally applicable purification procedures for DNA derivatives makes the synthesis of ESACHEL components amenable to robotization [for example using a TECAN Genesys 200-based workstation (TECAN, Männedorf, Switzerland), equipped with liquid handling system and a robotic manipulation arm]. Robotization may be necessary, in order to create ESACHEL sub-libraries containing several hundred different compounds.

[0065] The methodologies described in this Patent work not only for small organic molecules, but also for peptides and oligomeric proteins [e.g. antibody Fv fragments, consisting of a VH and VL domain; see Example 1]. Indeed, the attachment of a DNA heteroduplex at the C-terminus of cysteine-tagged VH and VL domains will provide an extra stabilization to the Fv heterodimer.

Biopanning experiments:

[0066] The use of ESACHEL for the identification of specific binders relies on the incubation of ESACHEL components with the target molecule (e.g., a protein of pharmacological interest), followed by the physical separation of the resulting complex from the ESACHEL components which have not bound to the target. In this respect, ESACHEL biopanning experiments are analogous to biopanning experiments which can be performed with phage libraries and/or ribosome display libraries, for which an extensive literature and several experimental protocols are available [Winter, 1994;; Viti, 2000; Schaffitzel, 1999]. For example, physical separation of the complex between ESACHEL members and the target molecule, from the pool of non-bound ESACHEL members, could be achieved by immobilizing the target molecule of a solid support (e.g. a plastic tube, a resin, magnetic beads, etc.).

The chelate effect:

[0067] Some of the contributions of ESACHEL technology for the identification of specific binders are related to a chemical process, termed the "chelate effect". The term chelate was first applied in 1920 by Sir Gilbert T. Morgan and H.D.K. Drew [J. Chem. Soc., 1920, 117, 1456], who stated: "The adjective chelate, derived from the great claw or chela (chely- Greek) of the lobster or other crustaceans, is suggested for the caliper-like groups which function as two associating units and fasten to the central atom so as to produce heterocyclic rings."

[0068] The chelate effect can be seen by comparing the reaction of a chelating ligand and a metal ion with the corresponding reaction involving comparable monodentate ligands. For example, comparison of the binding of 2,2'-bipyridine with pyridine or 1,2-diaminoethane (ethylenediamine) with ammonia. It has been known for many years that a comparison of this type always shows that the complex resulting from coordination with the chelating ligand is much more thermodynamically stable.

[0069] Let us consider the dissociation steps of a monodentate ligand, compared to multidentate (e.g., bidentate ligands). When a monodentate group is displaced, it is lost into the bulk of the solution. On the other hand, if one end of a bidentate group is displaced the other arm is still attached and it is only a matter of the arm rotating around and it can be reattached again (Figure 8). In general, the formation of the complex with bidentate groups is favored, compared to the complex with the corresponding monodentate groups.

[0070] The chelate effect has been shown to be able to contribute to high-affinity binding not only in the case of multidentate metal ligands, but in many other chemical situations, including binding interactions with macromolecules (e.g., multidentate DNA binding, chelating recombinant antibodies) [Neri D, Momo M, Prospero T, Winter G. High-affinity antigen binding by chelating recombinant antibodies (CRAbs). J Mol Biol. (1995) 246:367-73].

[0071] When examining some ESACHEL embodiments, for example those in which two chemical moieties are oligomerized by means of DNA heteroduplex formation, it is useful to illustrate the chelate effect in the context of the stability of the DNA heteroduplex which bridges the two chemical entities involved in the specific binding interaction with a target. In most cases, it will be convenient to have heteroduplexes (or triplexes or quadruplexes) which *de facto* do not dissociate in the experimental conditions chosen for the ESACHEL biopanning. Useful information and a discussion on the energetics of cooperative binding with short DNA heteroduplex fragments (8 bp) can be found in Distefano and Dervan, 1993 [Distefano MD, Dervan PB. Energetics of cooperative binding of oligonucleotides with discrete dimerization domains to DNA by triple helix formation Proc Natl Acad Sci USA. (1993) 90: 1179-1183.].

Considerations on the procedures following ESACHEL biopanning:

[0072] What happens after ESACHEL experiments, when specific binding members have been identified? For some purposes (e.g., certain biochemical experiments), it may be conceivable to use ESACHEL members without further chemical transformations. For example, one may want to measure binding affinities and kinetic constants for the binding of ESACHEL members to a target molecule.

[0073] For many applications, however; one may want to covert ESACHEL self-assembled molecules into analogues, in which the chemical entities responsible for the binding are covalently linked. The length, rigidity, stereoelectronic chemical properties and solubility of the linker will influence the binding affinity and performance of the resulting molecule [Shuker SB, Hajduk PJ, Meadows RP, Fesik SW. Discovering High-Affinity Ligands For Proteins - Sar by Nmr. Science (1996) 274:1531-1534] (see also Example 4).

EXAMPLES

Example 1:

[0074] As mentioned in previous sections, one strength of ESACHEL technology is its compatibility with a variety of different chemical moieties, including peptides and globular proteins (e.g., antibody domains).

[0075] In this example, we show how a simple embodiment of ESACHEL (Figure 1), featuring cysteine-tagged antibody variable domains covalently linked to DNA oligonucleotides capable of partial heteroduplex formation, leads to the identification of a pair of variable heavy domain (VH) and variable light domain (VL), which yield a specific antigen binding after heterodimerization.

[0076] The genes of the VH and VL domains of the L19 antibody (specific for the ED-B domain of fibronectin [Pini A, Viti F, Santucci A, Carnemolla B, Zardi L, Neri P, Neri D. Design and use of a phage display library. Human antibodies with sub-nanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. J Biol Chem. (1998) 273:21769-21776]), of the HyHEL-10 antibody (specific for hen egg lysozyme [Neri, 1995]; please note that an internal *Eco*RI site had previously been mutagenized without altering the protein sequence) and of other antibodies isolated from the ETH-2 library (Viti, 2000), are PCR amplified using the following pairs of primers, which code for a cysteine

residue, appended at the C-terminal extremity of each V domain:

L19 and ETH-2:

L19VH_Eco_fo

TTT CAC ACA GAA TTC ATT AAA GAG GAG AAA TTA ACT ATG GAG GTG CAG CTG TTG GAG TCT

L19VH_Hind_ba

TCA ATC TGA TTA AGC TTA GTG ATG GTG ATG GTG ATG ACA TCC ACC ACT CGA GAC GGT GAC CAG GGT

L19VL_Eco_fo

TTT CAC ACA GAA TTC ATT AAA GAG GAG AAA TTA ACT ATG GAA ATT GTG TTG ACG CAG TCT CCA

L19VL_Hind_ba

TCA ATC TGA TTA AGC TTA GTG ATG GTG ATG GTG ATG ACA TCC ACC TTT GAT TTC CAC CTT GGT CCC TTG

HyHEL-10:

HH10VH_Eco_fo

TTT CAC ACA GAA TTC ATT AAA GAG GAG AAA TTA ACT ATG GAG GTG AAG CTG CAG CAG TCT

HH10VH_Hind_ba

TCA ATC TGA TTA AGC TTA GTG ATG GTG ATG GTG ATG ACA TCC ACC TGC AGA GAC AGT GAC CAG AGT

HH10VL_Eco_fo

TTT CAC ACA GAA TTC ATT AAA GAG GAG AAA TTA ACT ATG GAT ATT GTG CTA ACT CAG TCT CCA

HH10VL_Hind_ba

TCA ATC TGA TTA AGC TTA GTG ATG GTG ATG GTG ATG ACA TCC ACC TTT TAT TTC CAG CTT GGT CCC CCC

[0077] The resulting PCR products are subcloned, using standard molecular biology procedures, into the *Eco*RI/*Hind*III sites of plasmid pQE12 (Qiagen, Germany). The resulting plasmids code for V domains, which carry the following sequence at their C-terminus: -Gly-Gly-Cys-His-His-His-His-His-His.

[0078] The plasmids, encoding the cysteine-tagged V-domains, are electroporated into *E.coli* cells (preferentially, in the Origami strain of Novagen, which have a slightly oxidizing cytoplasmic redox potential), expressed and purified, using metal chelate chromatography on NiNTA resin (Qiagen, Germany).

[0079] The cysteine-tagged V domains are reduced with 1 mM dithiothreitol solution in PBS (50 mM phosphate buffer + 100 mM NaCl, pH = 7.4), followed by desalting on a PD-10 column (Amersham-Pharmacia, Dübendorf, Switzerland).

[0080] In parallel, different oligonucleotides, carrying a thiol group at the 3' end or at the 5' end, are ordered from a commercial supplier (e.g., Microsynth, Balgach, Switzerland). Individual DNA oligonucleotides with the thiol group at the 3' end are used for coupling to individual VH domains. Individual DNA oligonucleotides with the thiol group at the 5' end are used for coupling to individual VL domains.

[0081] Representative sequence types are illustrated below. Please note that oligonucleotides of these families are capable of partial heteroduplex formation:

L19:

L19_5SH

5-HS-GGA GCT TCT GAA TTC TGT GTG CTG CAT AAT CGA CAC GAA TTC CGC AGC-3'

L19_3SH

5'-TCG CGA GGG GAA TTC GTC ATA TAT CAG CAC ACA GAA TTC AGA AGC TCC-SH-3'

HyHEL-10:

HyHel10_5SH

5-HS-GGA GCT TCT GAA TTC TGT GTG CTG CAG TGG CGA CAC GAA TTC CGC AGC-3'

HyHel10_3SH

5'-TCG CGA GGG GAA TTC GTC ATA GGG CAG CAC ACA GAA TTC AGA AGC TCC-SH-3'

Anti-GST (from ETH-2 library):

GST_5SH

5-HS-GGA GCT TCT GAA TTC TGT GTG CTG CTG AGG CGA CAC GAA TTC CGC AGC-3'

GST_3SH

5'-TCG CGA GGG GAA TTC GTC AAG AGG CAG CAC ACA GAA TTC AGA AGC TCC-SH-3'

[0082] In parallel reactions, the purified thiol-containing DNA oligonuclotides are reacted with a molar excess of bis-maleimido-hexane (Pierce, Belgium) in PBS + DMSO, following the manufacturer's instructions. The resulting derivatives are purified from unreacted bismaleimido-hexane using anion exchange chromatography, then reacted with slight molar excess of purified VH-cys or VL-cys, respectively, at a domain concentration > 0,1 mg/ml. The resulting (V domain)-DNA reaction products are separated from unreacted V-domain by anion exchange chromatography.

[0083] An equimolar mixture of (V domain)-DNA derivatives is mixed in PBS, heated at 70 degrees centigrade for 1 minute, then let equilibrate until it reaches room temperature. The resulting mixture of ESACHEL compounds is then incubated with a 0.1 μM solution of biotin-ED-B In PBS at room temperature for 10 minutes (Pini, 1998), then captured on streptavidin-coated magnetic beads and washed extensively according to standard procedures.

[0084] The resulting bead preparation is then used as template for two separate PCR reactions, which amplify the (L19_5SH, HyHel10_5SH, GST_5SH) and (L19_3SH, HyHel10_3SH, GST_3SH) oligonucleotides (see above), using oligos:

1AB_PCRfo
5'-GGA GCT TCT GAA TTC TGT GTG CTG-3'

1APCRba
5'-GCT GCG GAA TTC GTG TCG-3'

1B_PCRba
5'-TCG CGA GGG GAA TTC GTC-3'.

[0085] The resulting PCR products are digested with *Eco*R1, ligated to form concatenamers, subcloned into a suitable host plasmid, followed by electroporation in *E.coli* and sequencing. The resulting sequence analysis shows a strong bias towards L19 codes (CAT AAT and ATA TAT) over HyHEL-10 and GST codes, indicating a preferential enrichment of VH(L19)-VL(L19) combinations over the other possible assembly products.

Example 2:

[0086] In this example, we describe how the ESACHEL embodiment of Figure 1 can be performed in a practical implementation. The experimental strategy outlined here is also applicable to the embodiments described in Figure 4, in which DNA triplexes or DNA quadruplexes are used to display chemical entities at the extremity of self-assembling oligonucleotides.

Two sub-libraries are constructed as follows:

[0087] A sub-library "A" is created, by coupling n compounds to the 3' extremity of n different DNA oligonucleotides. Among the many different possible implementations, a convenient one is represented by the coupling of iodoacetamido- or maleimido-derivatives of n chemical entities to individual DNA oligonucleotides, which carry a thiol group at the 3' end. The coupling can easily be performed at room temperature in PBS (50 mM phosphate buffer + 100 mM NaCl, pH = 7.4), by simple mixing of the thiol-bearing oligonucleotide (typical concentration range: 10 - 100 μM) with a molar

excess of iodoacetamido- or maleimido-derivative (typical concentration range: 50 - 500 μM), followed by chromatographic purification of the DNA-chemical entity adduct. The thiol-containing oligonucleotides can be purchased from commercial suppliers. Each of them contains a constant sequence portion (e.g., 5'-XXXXXCAGCACACAGAATTCA-GAAGCTCC-3') capable of heteroduplex formation with members of sub-library B (see below). The DNA sequence portion XXXXX at the 5' end is (at least in part) different in each member of the sub-library A, therefore acting as a code.

[0088] Similarly, a sub-library "B" is created, by coupling m compounds to the 5' extremity of m different DNA oligonucleotides. Coupling of iodoacetamido- or maleimido-derivatives of m chemical entities to individual DNA oligonucleotides, which carry a thiol group at the 5' end, is performed similar to what described for sub-library "A". Such oligonucleotides can be purchased from commercial suppliers. Each of them contains a constant sequence portion (e.g., 5'-GGAGCTTCTGAATTCTGTGTGCTGYYYYY-3') capable of heteroduplex formation with members of sub-library A (see above). The DNA sequence portion YYYYY at the 3' end is (at least in part) different in each member of the sub-library B, therefore acting as a code.

[0089] Assembly of sub-library A members with sub-library B members is carried out by mixing the sub-libraries in PBS, heating the mixture at 70 degrees centigrade for 1 minute (if compatible with the stability of the chemical entities used in sub-library construction), followed by equilibration at room temperature. The resulting ESACHEL library contains n x m members, and can be used in biopanning experiments, followed by decoding of the binding members.

Example 3:

[0090] This example illustrates one of the many possible decoding methodologies, for ESACHEL embodiments as described in Figure 1 and in Example 2.

[0091] The decoding strategy, schematically depicted in Figure 5, is based on the principle that, after biopanning of desired ESACHEL binding specificities, PCR fragments are generated, each of which carries the code of pairs of sub-library members, whose combination was rescued in the biopanning experiment, therefore allowing the identification of the corresponding heterodimerized chemical entities.

[0092] Chemical entities of sub-libraries A and B (see also Figure 1 and Example 2) are coupled, individually, to members of two pools of DNA oligonucleotides with the following properties:

- One pool of oligonucleotides carries the chemical entities at the 3'-end (pool A), whereas the other pool carries the chemical entity at the 5'-end (pool B).
- A sufficient number of bases at the 5' extremity of oligonucleotides of pool B allow the specific dimerization of any individual member of pool B with any individual member of pool A. Inside this dimerization domain, oligonucleotides from pool B contain a "code" region, which codes for the chemical entity at the 5'-end. Oligonucleotides of pool A contain a sufficient number of desoxyribose backbone elements without bases (d-Spacer), to prevent any undesired pairing to the bases of code B.
- Oligonucleotides of sub-library A have their distinctive code towards the 5' extremity.

[0093] Oligonucleotides of sub-library B remain stably annealed to oligonucleotides of sub-library A, and can work as primers for a DNA polymerase reaction on the template A. The resulting DNA segment, carrying both code A and code B, can be amplified (typically by PCR), using primers which hybridize at the constant extremities of the DNA segment (Figure 5).

[0094] As an example of model oligonucleotides A and B which can be used for the generation of a PCR product, which carries both code A and B, is provided below:

typeB_oligo

Chemical entity B - 5'-GCA TAC CGG AAT TCC CAG CAT AAT GAT CGC TAT CGC TGC-3'

typeA_oligo (d=d-Spacer element)

5'-CGT CAG CTC GAA TTC TCC ATA TAT GCA GCG ATA GCG ATC DDD DDD CTG GGA ATT CCG GTA TGC -3' – chemical entity A

CodeABfo
5'-GCA TAC CGG AAT TCC CAG-3'
CodeABba
5'-CGT CAG CTC GAA TTC TCC-3'

[0095] typeA_oligo and type_B oligo are mixed in approx. equimolar amounts. The resulting mixture is heated up to 70°C, and cooled to room temperature, allowing the heterodimerization of typea_oligo and type_B oligo. The resulting mixture is mixed with a suitable buffer for PCR reaction, dNTPs (250 μM per nucleotide, Pharmacia). Taq-Polymerase (1U, Appligen) is then added, and followed by incubation of the mixture at 40°C for 5 minutes. Next, a PCR program with 30 cycles of (90°/1 minute)-(50°C/1 minute)- (72°C/15 seconds) is started after addition of primers CodeABfo and CodeABba (400μM). After completion of the program, the length of the PCR fragment is checked by standard polyacrylamide gel methodology, using commercial Novex gels. Its sequence identity can be established by digesting the product with *Eco*RI, followed by cloning into a suitable plasmid and sequencing.

Example 4:

[0096] Like chelating recombinant antibodies (CRAbs) [Neri, 1995] and small organic ligands identified using SAR by NMR [Shuker, 1996], the high-affinity binding of ESACHEL members to target molecules may rely on the chelate effect.

[0097] It is intuitive that the affinity gain contribution of the chelate effect will depend on the length, rigidity, stereoelectronic chemical properties and stability of the linkage between the two (or more) chemical entities, in contact with the target antigen. Furthermore, the affinity gain will directly depend on the magnitude of the association and dissociation rate constants (k_{on} and k_{off}) of the individual chemical entities, binding to the target.

[0098] In this example, we present a computational model, which provides information about the contribution of the chelate effect, in relation to the above mentioned parameters (linker length, k_{on} and k_{off}).

[0099] As depicted in Figure 9, two different chemical entities A and B bind to distinct binding sites of the same target molecule, and are connected by a linker of defined length. It is convenient to define four different states (nI, nII, nIII and nIV), which may interconvert by means of chemical binding equilibria:

nI: Both A and B bound to their binding pocket
nII: A bound to its binding pocket, while B is not bound to its binding pocket
nIII: B bound to its binding pocket, while A is not bound to its binding pocket
nIV: Both A and B not bound to their binding pockets

[0100] The kinetic parameters k_{onA}, k_{offA}, k_{onB} and k_{offB}, describing the binding properties of the individual chemical entities A and B to the corresponding binding pockets, are known. From these constants, it is possible to determine probabilities for a bound molecule to go off the binding pocket (poff), and for an unbound molecule to bind to its binding pocket (pon) in a defined time increment.

$$poff = k_{off} \bullet \Delta t \qquad [1]$$

[0101] In first order kinetics, the half life of binding can be expressed as:

$$\tau^{on} = \frac{\ln 2}{k_{on} \bullet [B]} \qquad [2]$$

[0102] If at time t=0 all molecules B are not bound to the corresponding binding pocket (and if one neglects in a first approximation dissociation processes), the fraction of bound molecules after the time increment Δt can be expressed as follows:

$$\frac{N(\Delta t)}{N(t=0)} = e^{-\Delta t \bullet k_{on} \bullet [B]} \qquad [3]$$

[0103] If one chooses a sufficiently large ensamble of molecules, the equation [3] can be approximated to the probability that a molecule B binds to its pocket in the time increment Δt.

[0104] The equations written so far correspond to chemical entities A and B, which bind to the corresponding pockets independent from one another. Let us assume, however, that A and B are connected by a linker, and that moiety A is bound to its target. It is convenient to express the concentration of B in the vicinity of its target binding pocket as effective concentration, ec.

$$pon = e^{-\Delta t \cdot k_{on} \cdot ec} \qquad [4]$$

[0105] In our model, the contribution of the chelate effect to the binding properties of the A-B bidentate molecule to the target is due to an increase of the effective concentration of one of the two binding moieties, when the other one is bound to its binding pocket (Figure 9). In a simple model, let us assume that, if binding molecule A is bound, the molecule B can be situated with equal likelihood in every position in a half spherical space around molecule A, whereby the radius "rad" (measured in meters) is equal to the linker length. Sterical constraints of the linker, repulsion effects etc. are neglected in this simple model. The same assumption is used when molecule B is bound, and A is unbound. As a result, the molar effective concentration ec can be computed as:

$$ec = \frac{1}{\left[\frac{1}{2} \cdot \left(\frac{4}{3} \cdot \pi \cdot rad^3\right) \cdot 6.01 \cdot 10^{26}\right]} \qquad [5]$$

[0106] Based on these assumptions, we designed a computer program to estimate the contribution of the chelate effect to the residual half life of a bidentate binding molecule A-B, where the two individual moieties A and B binding to two distinct binding pockets on the same target molecule are connected with a liker of the length rad. The possibility of A and B binding to two different target molecules is neglected.

[0107] In a population of n A-B molecules, the four states of Figure 9 can be populated by the individual molecules, and it is conceivable that individual molecules are found in different states at different times of observation. In our model, we determined the probabilities pon and poff of the individual molecules A and B to change their state within a time increment Δt.

[0108] As a practical example, let us consider that at time t=0, all molecules A-B are in state nI (both A and B are bound). At every time increment Δt (which is 1 second in the program) the probabilities of the moieties A and B to change their binding status give rise to a new distribution of molecules A-B in the four different states. In the simulation, irreversible dissociation conditions are used (i.e., dissociated molecules A-B, in state nIV are not allowed to bind back to the target). The program repeats these calculations time increment after time increment, until the population of molecules A-B bound to the target (sum of nI, nII and nIII) drops to half of the starting population. The sum of the time increments gives an estimate of the half-life of a bound molecule A-B to its target.

[0109] By varying either the initial configuration of the ensamble of molecules, or the parameters k_{offA}, k_{offB}, k_{onA}, k_{onB} and rad, one can estimate the contribution to the chelate effect of different linked chemical entities, in terms of the kinetic stabilization of the complex.

[0110] The code of the corresponding CHELATE programm (written in PASCAL) is listed below:

```
**************
program chelate;

var
n, n0, koffA, koffB, koffAB, konA, konB :double;
t12A, t12B, t12AB, rad, conc: double;
linkerA : integer;
nI, nII, nIII, nIV, deltaI, deltaII, deltaIII, deltaIV : double;
pAoff, pAon, pBoff, pBon : double;

begin
writeln;
```

```
writeln ('molecules A and B are connected with a linker');
writeln;

writeln ('koffA [s-1] = '); (* type in values *)
readln (koffA);
writeln ('konA [M-1s-1] = ');
readln (konA);
writeln ('koffB [s-1] = ');
  readln (koffB);
  writeln ('konB [s-1] = ');
  readln (konB);
  writeln;
  writeln ('linker length [A] =');
  readln (linkerA);

  t12A:=In(2)/koffA; (* calculate t12 and concentration *)
  t12B:=In(2)/koffB;
  rad:=IinkerA*1e-10;
  conc:=1/((2/3*Pi*rad*rad*rad)*6.01e26);

  writeln ('koffA=',koffA,' t12A=',t12A,' konA=',konA);
  writeln ('koffB=',koffB,' t12B=',t12B,' konB=',konB);
 wrfteln ('linker length [m]=',rad);
 writeln ('effective concentration [M]=',conc);

 writeln;

 t12AB:=0; (* Parameters *)
 n0:=1e10;
 nI:=n0; nII:=0; nIII:=0; nIV:=0; (*in this embodiment of the program, only
the nI state is populated at time 0 *)
 n:=nI + nII + nIII;
 pAoff:=koffA;
 pAon:=1-exp(-1*konA*conc);
 pBoff:=koffB;
 pBon:=1-exp(-i*konB*conc);

 while n>n0/2 do
  begin
  deltaI:=0; deltaII:=0; deltaIII:==0; deltaIV:=0;
  t12AB:=t12AB+1; (* one loop equals one second *)
 (*nI*)
 deltaIV :=deltaIV +(nI*pAoff*pBoff);
 deltaIII:=deltaIIH-(nI*pAoff*(1-pBoff));
 deltaII :=deltaII +(nI*(1-pAoff)*pBoff);
deltaI :=deltaI -(nI*pAoff*pBoff);
deltaI :=delta -(nI*pAoff*(i-pBoff));
deltaI : =deltal -(nI*(i-pAoff)*pBoff);

(* nII *)
deltaIII: = deltaIII +(nII*pAoff*pBon);
deltaIV :=deltaIV +(nII*pAoff*(1-pBon));
deltaI =deltaI +(nII*(1-pAoff)*pBon);
deltaII :=deltaII -(nII*pAoff*pBon);
deltaII :=deltaII -(nII*pAoff*(1-pBon));
deltaII :=deltaII -(nII*(1-pAoff)*pBon);

(* nIII *)
deltaII : = deltaII +(nIII*pAon*pBoff);
deltaI :=deltaI +(nIII*pAon*(1-pBoff));
deltaIV :=deltaIV +(nIII*(1-pAon)*pBoff);
deltaIII:=deltaIII-(nIII*pAon*pBoff);
deltaIII:=deltaIII-(nIII*pAon*(1-pBoff));
```

```
deltaIII:=deltaIII-(nIII*(1-pAon)*pBoff);

nI :=nI + deltaI;
nII :=nII + deltaII;
nIII:=nIII + deltaIII;
nIV :=nIV + deltaIV;

n: =nI+nII+nIII;
(* nIV is removed from the remaining population *)
 writeln (n,' ',t12AB);

 end;

writeln;
writeln ('koffA=',koffA,' t12A=',t12A,' konA=',konA);
writeln ('koffB=',koffB,' t12B=',t12B,' konB=',konB);
writeln ('linker length [m]=',rad);
writeln ('effective concentration [M]=',conc);
writeln;
writeln ('t12AB=',t12AB,' s');
writeln;

readln;
end.
***************
```

Example 5:

[0111] In many cases, it may be desirable to improve the affinity of an existing binder towards a target molecule (e.g. a pharmaceutical target). Towards, this goal, ESACHEL technology can be used as follows, i.e. omitting the "code" oligonucleotide sequence from the binder to be optimized. Let us suppose that the chemical moiety **p** binds to a target molecule (e.g., a protein) with an insufficient affinity. It will be convenient to link **p** to one extremity (e.g., the 5' end) of a suitable oligonucleotide, capable of self-assembly with other oligonucleotide derivatives (typically, by heteroduplex formation, as depicted in Figure 10).

[0112] For example, the chemical moiety **p** will be coupled to the 5' end of oligonucleotide 5' - 5'- GGA GCT TCT GAA TTC TGT GTG CTG -3'. It will then be convenient to chemically couple, in individual reactions, many different chemical moieties **q** at the 3' end of oligonucleotides, of general sequence 5' - XX.....XX - Y - CAG CAC ACA GAA TTC AGA AGC TCC - 3', whereas:

- the XX.....XX portion will be different for the different compounds;
- Y represents a biotinylated base analogue;
- the sequence 5' - CAG CAC ACA GAA TTC AGA AGC TCC - 3' will be identical in all cases, allowing the heteroduplex formation with the sequence 5' - GGA GCT TCT GAA TTC TGT GTG CTG - 3', chemically coupled to **p**, for all members of the ensamble of molecules **q.**

[0113] The resulting library will pair **p** with molecules **q**, each of which bears a distinctive oligonucleotide code. The self-assembled library can be submitted to biopanning, under conditions of suitable stringency. The binders rescued at the end of the biopanning procedure will be identified by means of their code. For example, the codes of the molecules **q**, which together with **p** give rise to high-affinity binders for the target molecule, can be read by hybridization to an oligonucleotide chip, in which the different positions are covered with oligonucleotides, which are complementary to the sequences XX.....XX of the members of the sub-library Q. The biotin moiety on members of sub-library Q will allow the detection of the binding events on the chip.

[0114] Candidate chemical moieties **q** will then be chemically linked to p, and the resulting conjugate will be used as a specific binder for the target molecule of interest.

Claims

1. A chemical library comprising combination reaction products of at least two chemical compounds, each one of these chemical compounds comprising:

a) a chemical moiety (p,q) potentially capable of performing a binding interaction with a single target molecule;
b) an oligonucleotide (b,b'), a part of which being a self-assembly moiety (m,m');
the chemical compounds being bound to each other by the shelf-assembly moieties (m,m') of their oligonucleotides (b,b'), **characterized in that** the combination reaction product is stable in the absence of said target molecule, wherein the oligonucleotides (b,b') of each one of the chemical compounds comprise a variable, unique coding sequence (b2,b2') individually coding for the identification of the particular chemical moiety (p,q).

2. The chemical library of claim 1, **characterized in that** the at least two chemical compounds each comprise a chemical group by which they are covalently linked together after the stable combination reaction product had been formed.

3. The chemical library according to one of claims 1 or 2, **characterized in that** the oligonucleotides (b,b') are covalently and directly linked to the chemical moieties (p,q).

4. The chemical library according to one of claims 1 or 2, **characterized in that** the oligonucleotides (b,b') further comprise a linking portion (b3,b3') which is situated between the self-assembly sequence (b1,b1') and the chemical moiety (p,q).

5. The chemical library according to one of claims 1 or 2, **characterized in that** the coding sequence (b2,b2') of the oligonucleotide (b,b') is situated between the chemical moiety (p,q) and the self-assembly sequence (b1,b1').

6. The chemical library according to one of the preceding claims, **characterized in that** the combination reaction product is a dimer, trimer or tetramer and **in that** its individual combinations of moieties are derived by forming heteroduplexes, heterotriplexes or heteroquadruplexes of the self-assembly sequences (b1,b1') of the oligonucleotides (b,b').

7. The chemical library according to claim 6, **characterized in that** it comprises individually encoded sub-libraries (A) and (B), whereas sub-library (A) comprises **n** compounds coupled to the 3' extremity of **n** different DNA oligonucleotides (b) and sub-library (B) comprises **m** compounds coupled to the 5' extremity of **m** different DNA oligonucleotides (b').

8. The chemical library according to claim 7, **characterized in that** in sub-library (A) or in sub-library (B) respectively, iodoacetamido- or maleimido-derivatives of **n** or **m** chemical entities have been coupled to individual DNA oligonucleotides which carry a thiol group at the 3' or 5' end.

9. The chemical library according to claim 7, **characterized in that** in sub-library (A) or in sub-library (B) respectively, amide derivatives - forming chemical structures such as -O-P(O)$_2$-O-(CH$_2$)n-NH-CO-R, where R may correspond to a number of different chemical entities, and n may range between 1 and 10 - have been coupled to the oligonucleotides carrying a phosphodiester bond at one extremity.

10. The chemical library according to one of claims 7 to 9, **characterized in that** in sub-library (A) the self-assembly sequence (b1) is interrupted by a d-spacer in opposite position to a code (B), the d-spacer preventing any undesired pairing to the bases of code (B) which encodes sub-library (B), whereas the oligonucleotide (b) of sub-library (A) has its distinctive code (A) towards the 5' extremity.

11. A method of biopanning ligands specific for target molecules, wherein a combination reaction product is incubated with a target molecule, the combination reaction product consisting of at least two chemical compounds, each one of these chemical compounds comprising:

a) a chemical moiety (p,q) potentially capable of performing a binding interaction with a single target molecule;
b) an oligonucleotide (b,b') a part of which being a self-assembly moiety (m,m');
wherein the chemical compounds are bound to each other by the self-assembly moieties (m,m') of their oligonucleotides (b,b'), **characterized in that** a chemical library of combination reaction products according to one of claims 1 to 10 is used for biopanning.

12. A method to identify a target molecule with a combination reaction product of a chemical library according to one of the claims 1 to 10 comprising a chemical moiety (p,q) capable of performing a binding interaction with this target molecule and further comprising an oligonucleotide (b,b'), **characterized in that** the combination reaction product

is bound to a target by biopanning according to claim 11.

13. The method of claim 12, **characterized in that** PCR-fragments are generated by polymerase chain reaction (PCR), each of which carries the code of pairs of sub-library members (A) and (B), whereas sub-library (A) comprises **n** compounds coupled to the 3' extremity of n different DNA oligonucleotides (b) and sub-library (B) comprises m compounds coupled to the 5' extremity of **m** different DNA oligonucleotides (b').

14. The method of claim 13, **characterized in that** in sub-library (A) or in sub-library (B) respectively, iodoacetamido- or maleimido derivatives of **n** or **m** chemical entities are coupled to individual DNA oligonucleotides, which carry a thiol group at the 3' or 5' end.

15. The method of claim 14, **characterized in that** in sub-library (A) the self-assembly sequence (b1) is interrupted by a d-spacer in opposite position to a code (B), the d-spacer preventing any undesired pairing to the bases of code (B) which encodes sub-library (B), whereas the oligonucleotide (b) of sub-library (A) has its distinctive code (A) towards the 5' extremity.

16. The method of one of claims 12 to 15, **characterized in that** the length of the PCR-fragments are checked and their sequence identity is established by digesting the PCR-fragments with a restriction site for a specific endopeptidase (e.g. *Eco*RI), followed by cloning into a suitable plasmid and sequencing.

17. The method of one of claims 12 to 16 where several specific binding members are isolated at the end of a biopanning experiment, **characterized in that** concatenamers are created, starting from the various PCR-fragments present in the reaction mixture, the concatenated sequences are "read" by sequencing, revealing both the identity and the frequency of pairs of code (A) and code (B).

18. The method of claim 12 where several specific binding members are isolated at the end of a biopanning experiment and sub-libraries (A) and/or (B) carry chemical moieties at the extremities of partially-annealing oligonucleotides **characterized in that** unpaired DNA strands are hybridized with target oligonucleotides (e.g. DNA oligonucleotides) being immobilized on one or more chips.

19. The method of claim 18, **characterized in that** by using chip (A) or chip (B) respectively, the reading of the identity and/or frequency of members of sub-library (A) or sub-library (B) respectively, rescued after a biopanning experiment, is carried out and by decoding on chip (A) and (B) candidate components of sub-libraries (A) and (B), to be re-annealed and screened in a successive round of bio-panning are suggested.

20. The method of claim 19, **characterized in that** increasingly stringent binding to the target is mirrored by a reduction in the number of (A) and/or (B) members as identified on the respective chip and the possible combinations of the candidate (A) and (B) members are assembled individually or in smaller pools and assayed for binding to the target.

21. The method of one of the claims 18 to 20, **characterized in that** libraries are allowed to self-assemble in order to form trimeric or tetrameric complexes by using three or four chips, respectively, which carry distinctive target oligonucleotides for decoding.

22. The method of one of the claims 18 to 21, **characterized in that** the DNA of selected binding moieties is PCR amplified prior to chip hybridization.

Patentansprüche

1. Chemische Bibliothek, die Kombinationsreaktionsprodukte von mindestens zwei chemischen Verbindungen umfasst, wobei jede dieser chemischen Verbindungen umfasst:

   a) eine chemische Komponente (p,q), die potenziell fähig ist, eine Bindungs-Wechselwirkung mit einem einzelnen Zielmolekül auszuüben;
   b) ein Oligonukleotid (b,b'), von dem ein Teil eine Selbst-Zusammenfügungs-Komponente (m,m') ist;
   wobei die chemischen Verbindungen durch die Selbst-Zusammenfügungs-Komponenten (m,m') ihrer Oligonukleotide (b,b') aneinander gebunden sind, **dadurch gekennzeichnet, dass** das Kombinationsreaktionsprodukt in Abwesenheit des Zielmoleküls stabil ist, wobei die Oligonukleotide (b,b') jeder der chemischen Verbindungen

eine variable, eindeutige Codierungssequenz (b2,b2') umfassen, die individuell zur Identifizierung der bestimmten chemischen Komponente (p,q) codiert.

2. Chemische Bibliothek gemäss Anspruch 1, **dadurch gekennzeichnet, dass** die mindestens zwei chemischen Verbindungen jeweils eine chemische Gruppe umfassen, durch die sie kovalent miteinander verbunden werden, nachdem sich das stabile Kombinationsreaktionsprodukt gebildet hat.

3. Chemische Bibliothek gemäss einem der Ansprüche 1 oder 2, **dadurch gekennzeichnet, dass** die Oligonukleotide (b, b') kovalent und unmittelbar mit den chemischen Komponenten (p,q) verbunden sind.

4. Chemische Bibliothek gemäss einem der Ansprüche 1 oder 2, **dadurch gekennzeichnet, dass** die Oligonukleotide (b,b') ferner einen Verbindungsabschnitt (b3,b3') umfassen, der zwischen der Selbst-Zusammenfügungs-Sequenz (b1,b1') und der chemischen Komponente (p,q) angeordnet ist.

5. Chemische Bibliothek gemäss einem der Ansprüche 1 oder 2, **dadurch gekennzeichnet, dass** die Codierungssequenz (b2,b2') des Oligonukleotids (b, b') zwischen der chemischen Komponente (p,q) und der Selbst-Zusammenfügungs-Sequenz (b1,b1') angeordnet ist.

6. Chemische Bibliothek gemäss einem der vorangehenden Ansprüche, **dadurch gekennzeichnet, dass** das Kombinationsreaktionsprodukt ein Dimer, Trimer oder Tetramer ist, und dass seine individuellen Komponenten-Kombinationen durch die Bildung von Heteroduplexen, Heterotriplexen oder Heteroquadruplexen der Selbst-Zusammenfügungs-Sequenzen (b1,b1') der Oligonukleotide (b,b') gewonnen werden.

7. Chemische Bibliothek gemäss Anspruch 6, **dadurch gekennzeichnet, dass** diese individuell codierte Teil-Bibliotheken (A) und (B) umfasst,
wobei die Teil-Bibliothek (A) **n** Verbindungen umfasst, die mit dem 3'-Ende von **n** unterschiedlichen DNA-Oligonukleotiden (b) gekoppelt sind, und die Teil-Bibliothek (B) **m** Verbindungen umfasst, die mit dem 5'-Ende von **m** unterschiedlichen DNA-Oligonukleotiden (b') gekoppelt sind.

8. Chemische Bibliothek gemäss Anspruch 7, **dadurch gekennzeichnet, dass** in der Teil-Bibliothek (A) bzw. in der Teil-Bibliothek (B) Iodacetamid- oder Maleimid-Derivate von **n** oder **m** chemischen Einheiten an individuelle DNA-Oligonukleotide gekoppelt worden sind, die eine Thiolgruppe am 3'- oder 5'-Ende aufweisen.

9. Chemische Bibliothek gemäss Anspruch 7, **dadurch gekennzeichnet, dass** in der Teil-Bibliothek (A) bzw. in der Teil-Bibliothek (B) Amid-Derivat-bildende chemische Strukturen, wie beispielsweise -O-P(O)$_2$-O-(CH$_2$)n-NH-CO-R, wobei R einer Anzahl unterschiedlicher chemischer Einheiten entsprechen und n 1 bis 10 betragen kann, mit den Oligonukleotiden gekoppelt worden sind, die an einem Ende eine Phosphodiester-Bindung aufweisen.

10. Chemische Bibliothek gemäss einem der Ansprüche 7 bis 9, **dadurch gekennzeichnet, dass** die Selbst-Zusammenfügungs-Sequenz (b1) in der Teil-Bibliothek (A) durch einen, einem Code (B) gegenüber liegenden, d-Abstandhalter unterbrochen wird, wobei der d-Abstandhalter jede unerwünschte Paarbildung mit den Basen des die Teil-Bibliothek (B) codierenden Codes (B) verhindert, wohingegen das Oligonukleotid (b) der Teil-Bibliothek (A) seinen unverwechselbaren Code (A) gegen das 5'-Ende hin aufweist.

11. Verfahren zum biologischen Identifizieren ("Biopanning") von für Zielmoleküle spezifischen Liganden, bei welchem ein Kombinationsreaktionsprodukt mit einem Zielmolekül inkubiert wird und das Kombinationsreaktionsprodukt aus mindestens zwei chemischen Verbindungen besteht, wobei jede dieser chemischen Verbindungen umfasst:

a) eine chemische Komponente (p,q), die potenziell fähig ist, eine Bindungs-Wechselwirkung mit einem einzelnen Zielmolekül auszuüben;
b) ein Oligonukleotid (b,b'), von dem ein Teil eine Selbst-Zusammenfügungs-Komponente (m,m') ist;

wobei die chemischen Verbindungen durch die Selbst-Zusammenfügungs-Komponenten (m,m') ihrer Oligonukleotide (b,b') aneinander gebunden sind, **dadurch gekennzeichnet, dass** eine chemische Bibliothek von Kombinationsreaktionsprodukten gemäss einem der Ansprüche 1 bis 10 zum biologischen Identifizieren ("Biopanning") verwendet wird.

12. Verfahren zur Identifizierung eines Zielmoleküls mit einem Kombinationsreaktionsprodukt einer chemischen Biblio-

thek gemäss einem der Ansprüche 1 bis 10, das eine chemische Komponente (p,q) umfasst, die zum Ausüben eine Bindungs-Wechselwirkung mit diesem Zielmolekül befähig ist, und das ferner ein Oligonukleotid (b,b') umfasst, **dadurch gekennzeichnet, dass** das Kombinationsreaktionsprodukt mittels biologischem Identifizieren ("Biopanning") gemäss Anspruch 11 an ein Ziel gebunden ist.

13. Verfahren gemäss Anspruch 12, **dadurch gekennzeichnet, dass** mittels einer Polymerase-Kettenreaktion (PCR) PCR-Fragmente generiert werden, von denen ein jedes den Code von Paaren der Teil-Bibliothek-Mitglieder (A) und (B) aufweist, wobei die Teil-Bibliothek (A) **n** Verbindungen umfasst, die an die 3'-Enden von **n** unterschiedlichen DNA-Oligonukleotiden (b) gekoppelt sind, und die Teil-Bibliothek (B) **m** Verbindungen umfasst, die mit den 5'-Enden von **m** unterschiedlichen DNA-Oligonukleotiden (b') gekoppelt sind.

14. Verfahren gemäss Anspruch 13, **dadurch gekennzeichnet, dass** in der Teil-Bibliothek (A) bzw. in der Teil-Bibliothek (B) Iodacetamid- oder Maleimid-Derivate von **n** oder **m** chemischen Einheiten an individuelle DNA-Oligonukleotide gekoppelt sind, die eine Thiolgruppe am 3'-oder 5'-Ende aufweisen.

15. Verfahren gemäss Anspruch 14, **dadurch gekennzeichnet, dass** die Selbst-Zusammenfügungs-Sequenz (b1) in der Teil-Bibliothek (A) durch einen, einem Code (B) gegenüber liegenden, d-Abstandhalter unterbrochen wird, wobei dieser d-Abstandhalter jede unerwünschte Paarbildungen mit den Basen des die Teil-Bibliothek (B) codierenden Codes (B) verhindert, wohingegen das Oligonukleotid (b) der Teil-Bibliothek (A) seinen unverwechselbaren Code (A) gegen das 5'-Ende hin aufweist.

16. Verfahren gemäss einem der Ansprüche 12 bis 15, **dadurch gekennzeichnet, dass** die Länge der PCR-Fragmente geprüft und deren Sequenzidentität festgestellt wird, indem die einen Restriktionsort für eine spezifische Endopeptidase (z.B. *Eco*RI) aufweisenden PCR-Fragmente aufgeschlossen und anschliessend für ein geeignetes Plasmid kloniert und sequenziert werden.

17. Verfahren gemäss einem der Ansprüche 12 bis 16, bei dem einige spezifische Bindungsteilnehmer am Ende eines Biopanning-Experiments isoliert werden, **dadurch gekennzeichnet, dass** ausgehend von den unterschiedlichen im Reaktionsgemisch vorhandenen PCR-Fragmenten Koncatenamere erzeugt werden, wobei die koncatenierten Sequenzen, durch Sequenzieren "gelesen" werden, wodurch die Identität und die Häufigkeit von Paaren der Codes (A) und (B) offenbart wird.

18. Verfahren gemäss Anspruch 12, wobei am Ende eines Biopanning-Experiments mehrere spezifische Bindungsteilnehmer isoliert werden und Teil-Bibliotheken (A) und/oder (B) an den Enden sich teilweise wieder verbindender Oligonukleotide chemische Komponenten aufweisen, **dadurch gekennzeichnet, dass** ungepaarte DNA-Stränge mit Ziel-Oligonukleotiden (z.B. DNA-Oligonukleotiden) hybridisiert werden, die auf einem oder mehreren Chips immobilisiert sind.

19. Verfahren gemäss Anspruch 18, **dadurch gekennzeichnet, dass** durch die Verwendung des Chips (A) bzw. des Chips (B) das Lesen der Identität und/oder Häufigkeit von nach einem Biopanning-Experiment geborgen Mitgliedern der Teil-Bibliothek (A) bzw. der Teil-Bibliothek (B) durchgeführt wird, und dass durch Decodieren auf Chip (A) und (B) die Komponenten-Kandidaten der Teil-Bibliotheken (A) und (B) vorgeschlagen werden, welche in einer anschliessenden Biopanning-Runde wieder verbunden und ausgelesen werden sollen.

20. Verfahren gemäss Anspruch 19, **dadurch gekennzeichnet, dass** eine zunehmend starke Bindung an das Ziel durch eine Reduzierung der Anzahl der Mitglieder (A) und/oder (B) gemäss deren Identifizierung auf dem entsprechenden Chip widerspiegelt wird und die möglichen Kombinationen der Mitgliederkandidaten (A) und (B) einzeln oder in kleineren Pools zusammengefügt und hinsichtlich der Bindung an das Ziel untersucht werden.

21. Verfahren gemäss einem der Ansprüche 18 bis 20, **dadurch gekennzeichnet, dass** es Bibliotheken erlaubt wird sich selbst zusammenzufügen, um durch die Verwendung von drei bzw. vier charakteristische Ziel-Oligonukleotide für die Decodierung aufweisenden Chips, Trimer- oder Tetramer-Komplexe zu bilden.

22. Verfahren gemäss einem der Ansprüche 18 bis 21, **dadurch gekennzeichnet, dass** die DNA ausgewählter Bindungskomponenten vor der Chip-Hybridisierung mittels PCR vervielfacht wird.

Revendications

1. Bibliothèque chimique comprenant des produits de réaction de combinaison d'au moins deux composés chimiques, chacun de ces composés chimiques comprenant :

   a) une fraction chimique (p,q) potentiellement capable de réaliser une interaction de liaison avec une molécule cible unique ;
   b) un oligonucléotide (b,b'), dont une partie est une fraction d'auto-assemblage (m,m') ;
   les composés chimiques étant liés l'un à l'autre par les fractions d'auto-assemblage (m,m') de leurs oligonucléotides (b,b'), **caractérisée en ce que** le produit de réaction de combinaison est stable en l'absence de ladite molécule cible, les oligonucléotides (b,b') de chacun des composés chimiques comprenant une séquence codante variable unique (b2,b2') individuellement codante pour l'identification de la fraction chimique particulière (p,q).

2. Bibliothèque chimique selon la revendication 1, **caractérisée en ce que** lesdits au moins deux composés chimiques comprennent chacun un groupe chimique par lequel ils sont liés ensemble de façon covalente après que le produit de réaction de combinaison stable a été formé.

3. Bibliothèque chimique selon l'une des revendications 1 ou 2, **caractérisée en ce que** les oligonucléotides (b,b') sont liés de façon covalente et directe aux fractions chimiques (p,q).

4. Bibliothèque chimique selon l'une des revendications 1 ou 2, **caractérisée en ce que** les oligonucléotides (b,b') comprennent en outre une partie de liaison (b3,b3') qui est située entre la séquence d'auto-assemblage (b1,b1') et la fraction chimique (p,q).

5. Bibliothèque chimique selon l'une des revendications 1 ou 2, **caractérisée en ce que** la séquence codante (b2,b2') de l'oligonucléotide (b,b') est située entre la fraction chimique (p,q) et la séquence d'auto-assemblage (b1,b1').

6. Bibliothèque chimique selon l'une des revendications précédentes, **caractérisée en ce que** le produit de réaction de combinaison est un dimère, un trimère ou un tétramère et **en ce que** ses combinaisons individuelles de fractions sont dérivées en formant des hétéroduplex, des hétérotriplex ou des hétéroquadruplex des séquences d'auto-assemblage (b1,b1') des oligonucléotides (b,b').

7. Bibliothèque chimique selon la revendication 6, **caractérisée en ce qu'**elle comprend des sous-bibliothèques codées individuellement (A) et (B), la sous-bibliothèque (A) comprenant **n** composés couplés à l'extrémité 3' de **n** oligonucléotides d'ADN différents (b) et la sous-bibliothèque (B) comprenant **m** composés couplés à l'extrémité 5' de **m** oligonucléotides d'ADN différents (b').

8. Bibliothèque chimique selon la revendication 7, **caractérisée en ce que** dans la sous-bibliothèque (A) ou dans la sous-bibliothèque (B) respectivement, des dérivés iodoacétamido ou maléimido de **n** ou **m** entités chimiques ont été couplés à des oligonucléotides d'ADN individuels qui portent un groupe thiol à l'extrémité 3' ou 5'.

9. Bibliothèque chimique selon la revendication 7, **caractérisée en ce que** dans la sous-bibliothèque (A) ou dans la sous-bibliothèque (B) respectivement, des dérivés amide - formant des structures chimiques telles que -O-P $(O)_2$-O-(CH_2)n-NH-CO-R, où R peut correspondre à un certain nombre d'entités chimiques différentes, et où n peut se situer entre 1 et 10 - ont été couplés aux oligonucléotides portant une liaison phosphodiester à une extrémité.

10. Bibliothèque chimique selon l'une des revendications 7 à 9, **caractérisée en ce que** dans la sous-bibliothèque (A) la séquence d'auto-assemblage (b1) est interrompue par un d-espaceur en position opposée à un code (B), le d-espaceur empêchant tout appariement indésirable aux bases du code (B) qui code la sous-bibliothèque (B), tandis que l'oligonucléotide (b) de la sous-bibliothèque (A) a son code distinctif (A) vers l'extrémité 5'.

11. Procédé de sélection « *biopanning* » de ligands spécifiques pour des molécules cibles, dans lequel un produit de réaction de combinaison est incubé avec une molécule cible, le produit de réaction de combinaison étant constitué d'au moins deux composés chimiques, chacun de ces composés chimiques comprenant :

    a) une fraction chimique (p,q) potentiellement capable de réaliser une interaction de liaison avec une molécule cible unique ;

b) un oligonucléotide (b,b'), dont une partie est une fraction d'auto-assemblage (m,m') ;
les composés chimiques étant liés l'un à l'autre par les fractions d'auto-assemblage (m,m') de leurs oligonucléotides (b,b'), **caractérisé en ce qu'**une bibliothèque chimique de produits de réaction de combinaison selon l'une des revendications 1 à 10 est utilisée pour la sélection « *biopanning* ».

12. Procédé d'identification d'une molécule cible avec un produit de réaction de combinaison d'une bibliothèque chimique selon l'une des revendications 1 à 10 comprenant une fraction chimique (p,q) capable de réaliser une interaction de liaison avec cette molécule cible et comprenant en outre un oligonucléotide (b,b'), **caractérisé en ce que** le produit de réaction de combinaison est lié à une cible par sélection « *biopanning* » selon la revendication 11.

13. Procédé selon la revendication 12, **caractérisé en ce que** des fragments de PCR sont générés par réaction en chaîne par polymérase (PCR), dont chacun porte le code de paires d'éléments de sous-bibliothèques (A) et (B), la sous-bibliothèque (A) comprenant **n** composés couplés à l'extrémité 3' de **n** oligonucléotides d'ADN différents (b) et la sous-bibliothèque (B) comprenant **m** composés couplés à l'extrémité 5' de **m** oligonucléotides d'ADN différents (b').

14. Procédé selon la revendication 13, **caractérisé en ce que** dans la sous-bibliothèque (A) ou dans la sous-bibliothèque (B) respectivement, des dérivés iodoacétamido ou maléimido de **n** ou **m** entités chimiques sont couplés à des oligonucléotides d'ADN individuels, qui portent un groupe thiol à l'extrémité 3' ou 5'.

15. Procédé selon la revendication 14, **caractérisé en ce que** dans la sous-bibliothèque (A) la séquence d'auto-assemblage (b1) est interrompue par un d-espaceur en position opposée à un code (B), le d-espaceur empêchant tout appariement indésirable aux bases du code (B) qui code la sous-bibliothèque (B), tandis que l'oligonucléotide (b) de la sous-bibliothèque (A) a son code distinctif (A) vers l'extrémité 5'.

16. Procédé selon l'une des revendications 12 à 15, **caractérisé en ce que** la longueur des fragments de PCR est contrôlée et leur identité de séquence est établie par digestion des fragments de PCR avec un site de restriction pour une endopeptidase spécifique (par ex. *Eco*RI), suivie d'un clonage dans un plasmide approprié et d'un séquençage.

17. Procédé selon l'une des revendications 12 à 16 où plusieurs éléments de liaison spécifiques sont isolés à la fin d'une expérience de sélection « *biopanning* », **caractérisé en ce que** des concatémères sont créés, à partir des divers fragments de PCR présents dans le mélange réactionnel, les séquences concaténées sont « lues » par séquençage, révélant à la fois l'identité et la fréquence des paires de code (A) et de code (B).

18. Procédé selon la revendication 12 où plusieurs éléments de liaison spécifiques sont isolés à la fin d'une expérience de sélection « *biopanning* » et où les sous-bibliothèques (A) et/ou (B) portent des fractions chimiques aux extrémités d'oligonucléotides à appariement partiel, **caractérisé en ce que** des brins d'ADN non appariés sont hybridés avec des oligonucléotides cibles (par ex. des oligonucléotides d'ADN) immobilisés sur une ou plusieurs puces.

19. Procédé selon la revendication 18, **caractérisé en ce que,** en utilisant la puce (A) ou la puce (B) respectivement, la lecture de l'identité et/ou fréquence d'éléments de la sous-bibliothèque (A) ou sous-bibliothèque (B) respectivement, récupérés après une expérience de sélection « *biopanning* », est effectuée et par décodage sur les puces (A) et (B) des composants candidats des sous-bibliothèques (A) et (B), destinés à être réappariés et criblés dans un cycle successif de sélection « *biopanning* », sont suggérés.

20. Procédé selon la revendication 19, **caractérisé en ce qu'**une liaison à la cible dans des conditions de plus en plus sévères se traduit par une réduction du nombre d'éléments (A) et/ou (B) tels qu'identifiés sur la puce respective et les combinaisons possibles d'éléments candidats (A) et (B) sont assemblées individuellement ou dans des pools plus petits et testées pour leur liaison à la cible.

21. Procédé selon l'une des revendications 18 à 20, **caractérisé en ce qu'**on laisse des bibliothèques s'auto-assembler afin de former des complexes trimères ou tétramères en utilisant trois ou quatre puces, respectivement, qui portent des oligonucléotides cibles distinctifs pour le décodage.

22. Procédé selon l'une des revendications 18 à 21, **caractérisé en ce que** l'ADN de fractions de liaison sélectionnées est amplifié par PCR avant l'hybridation sur puce.

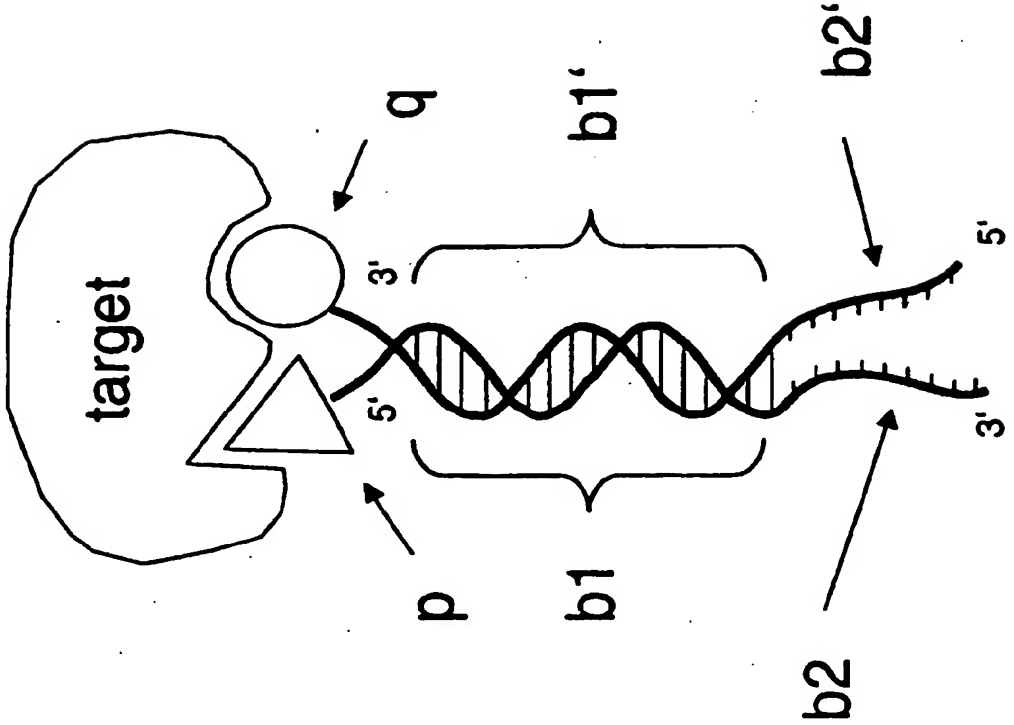
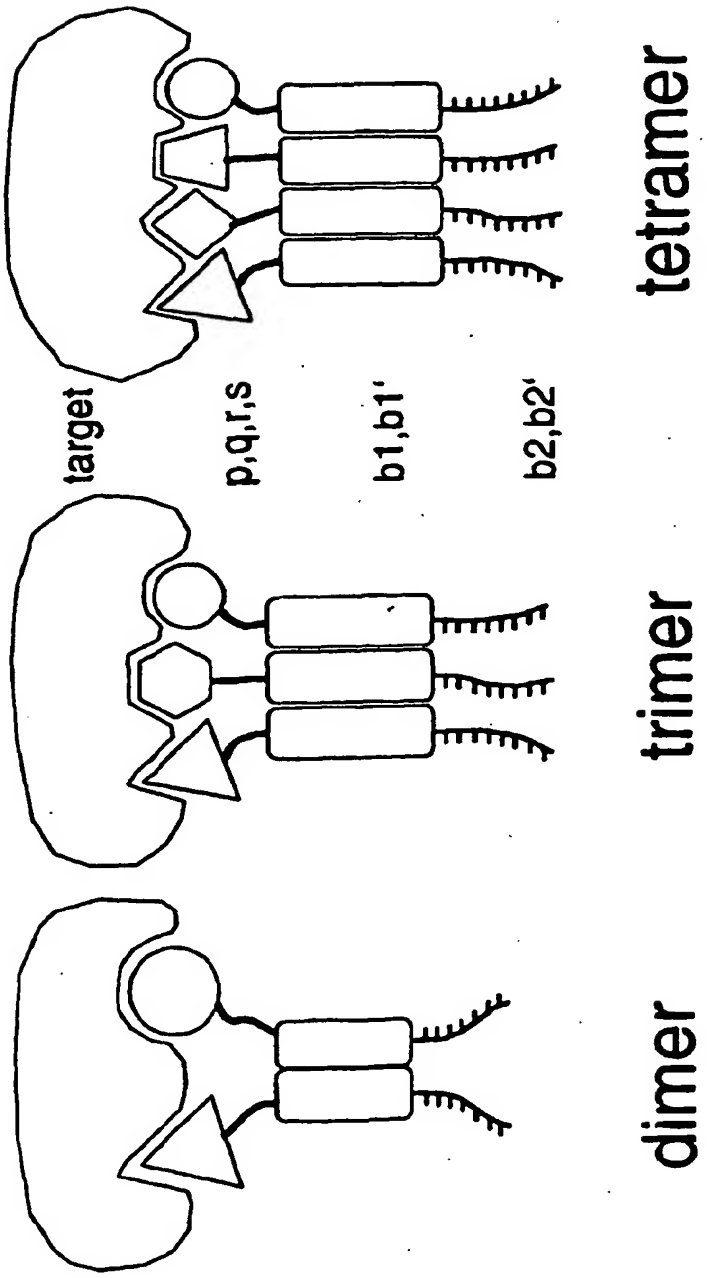


Fig. 1

Fig. 2



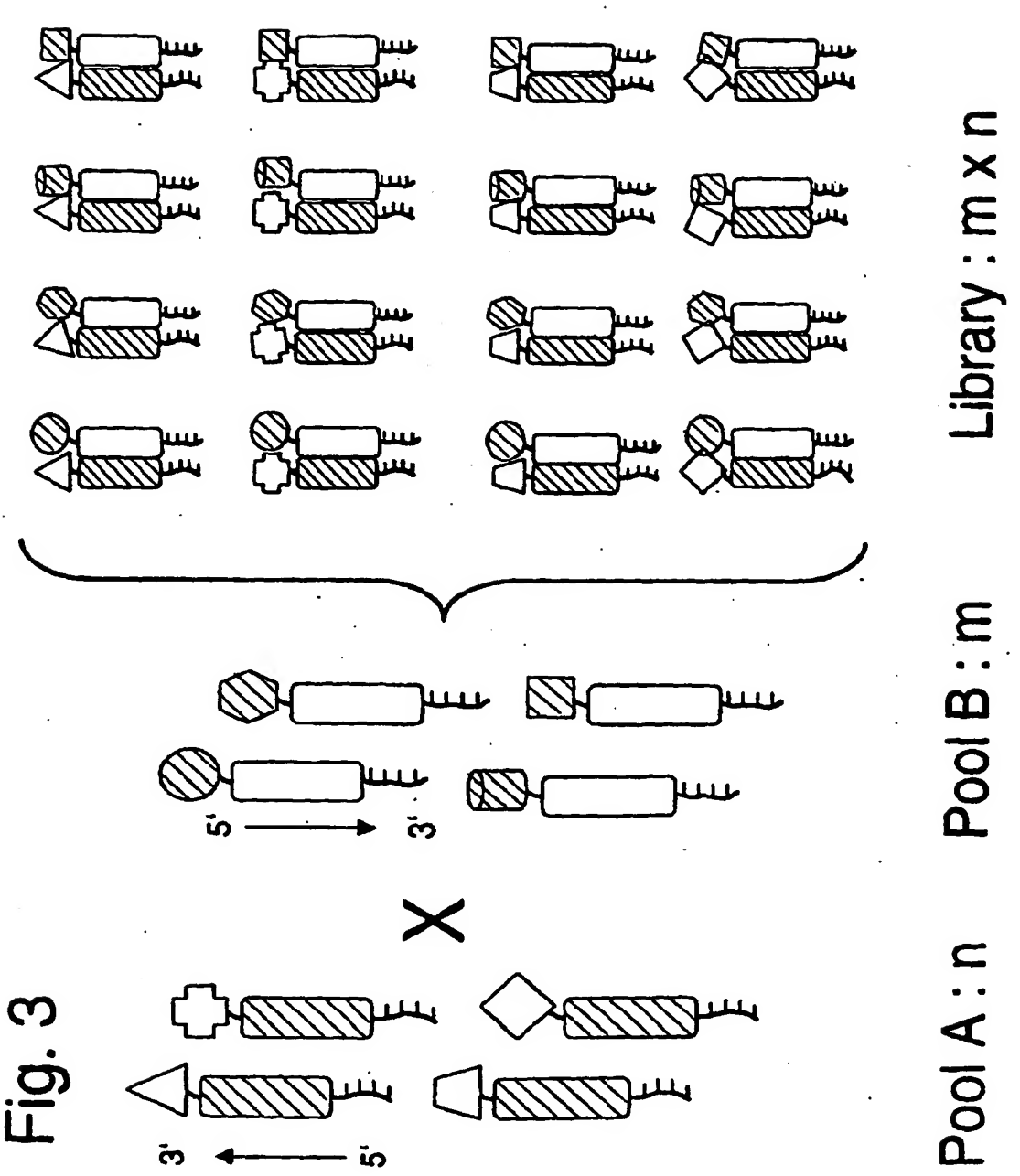


Fig. 3

Fig. 4

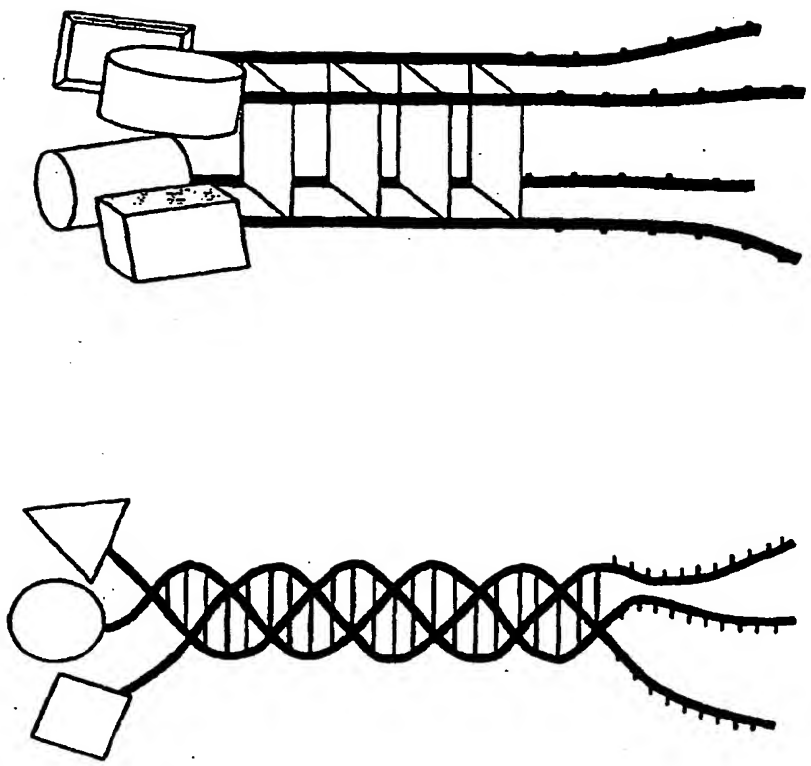


Fig. 5

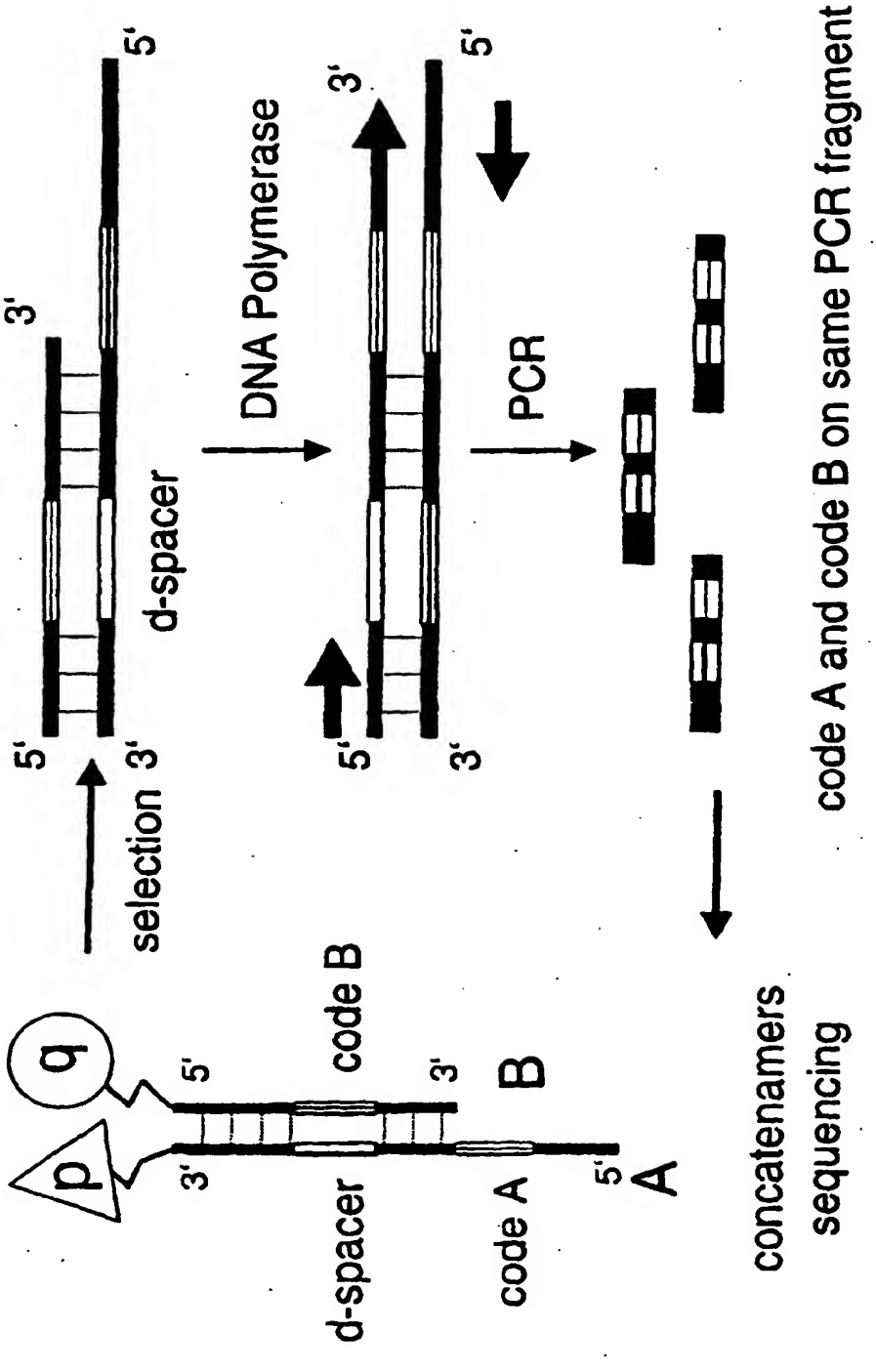

q
p
5'
3'
code B
d-spacer
code A
B
A
selection
DNA Polymerase
PCR
concatenamers
sequencing
code A and code B on same PCR fragment

Fig. 6

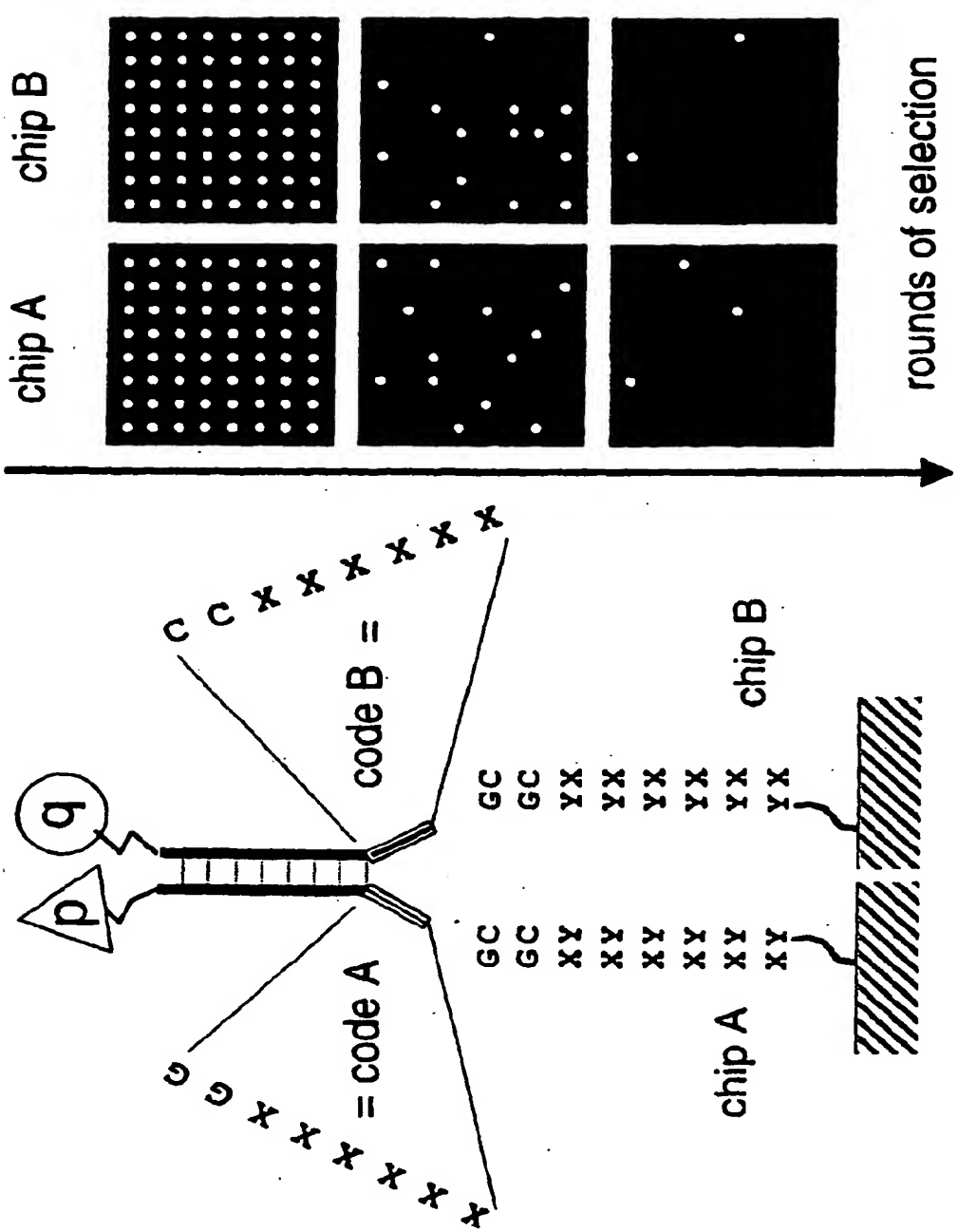


Fig. 7

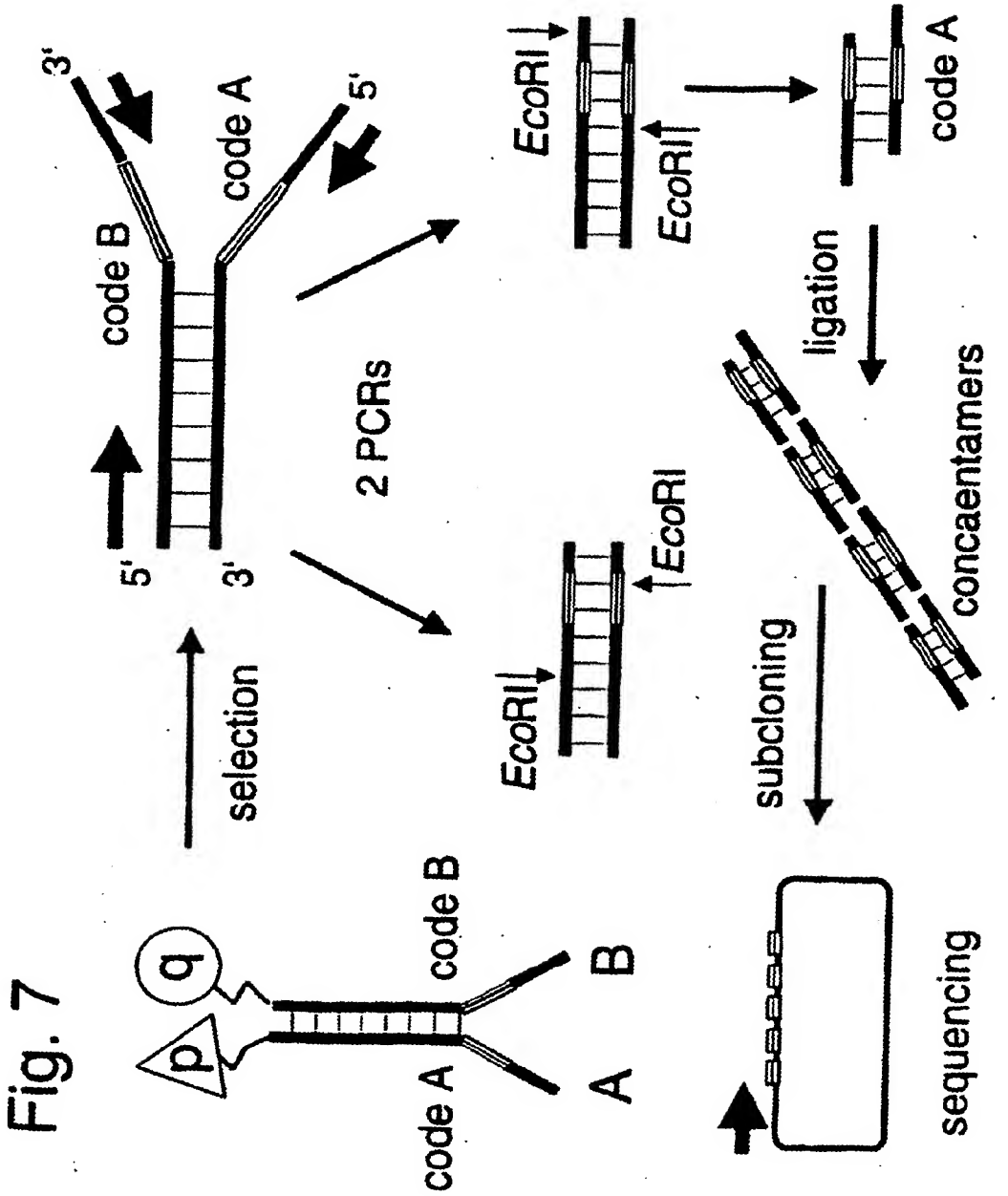

3'
code B
code A
5'
5'
3'
selection
2 PCRs
EcoRI
EcoRI
EcoRI
EcoRI
code A
ligation
concaentamers
subcloning
q
p
code B
code A
A
B
sequencing

Fig. 8

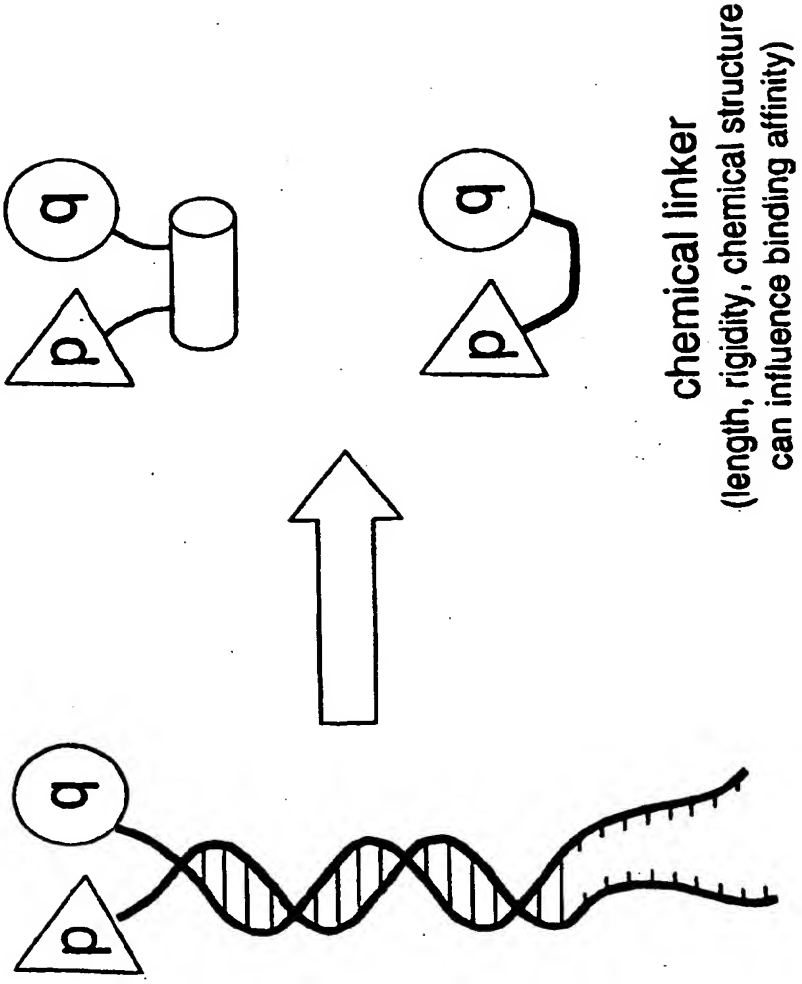
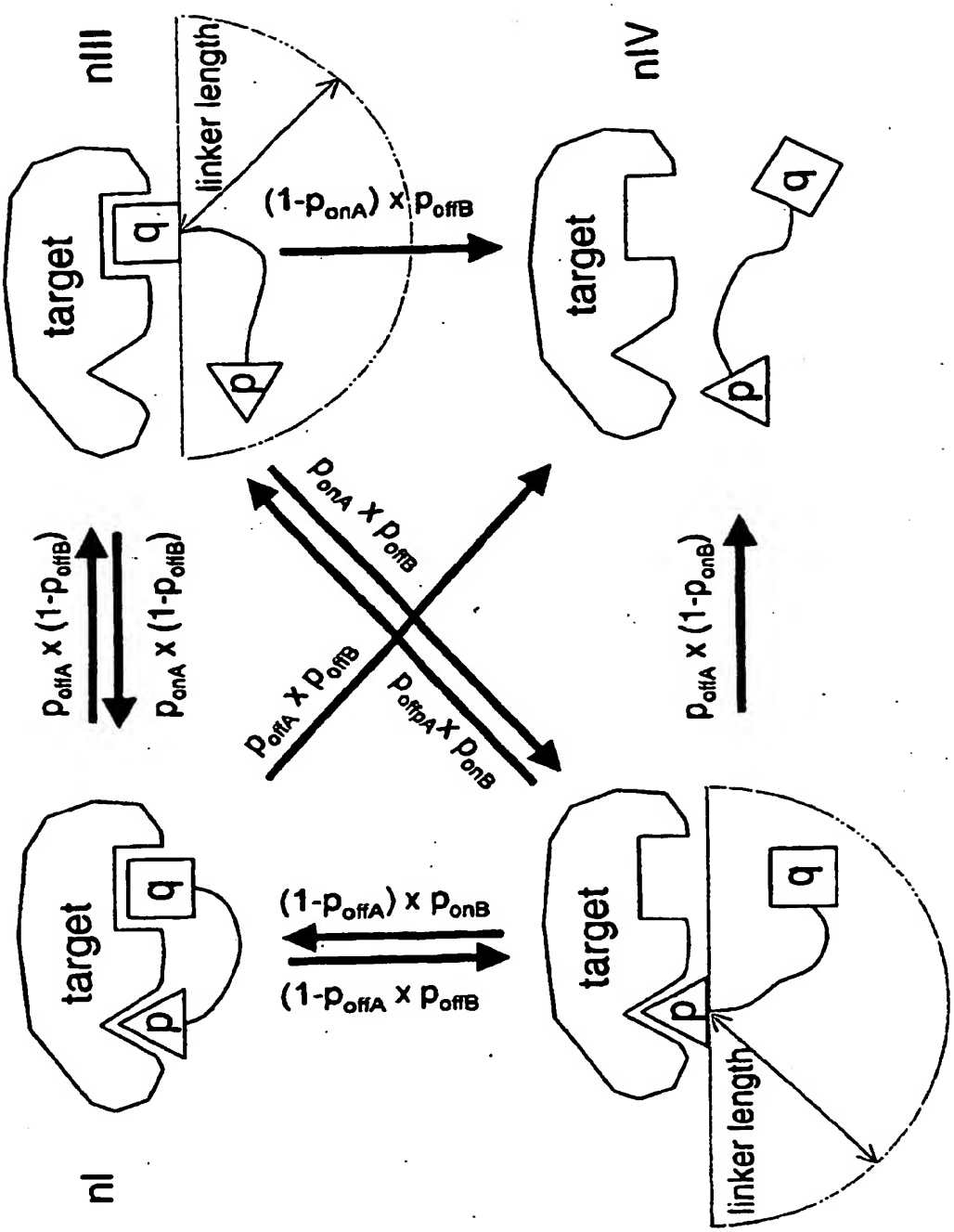

q
p
q
p
chemical linker
(length, rigidity, chemical structure
can influence binding affinity)
q
p

Fig. 9


nI
nII
nIII
nIV
target
q
p
linker length
$(1-p_{offA}) \times p_{onB}$
$(1-p_{offA} \times p_{offB}$
$(1-p_{onA}) \times p_{offB}$
$p_{offA} \times (1-p_{offB})$
$p_{onA} \times (1-p_{offB})$
$p_{onA} \times p_{offB}$
$p_{offA} \times p_{offB}$
$p_{offpA} \times p_{onB}$
$p_{offA} \times (1-p_{onB})$

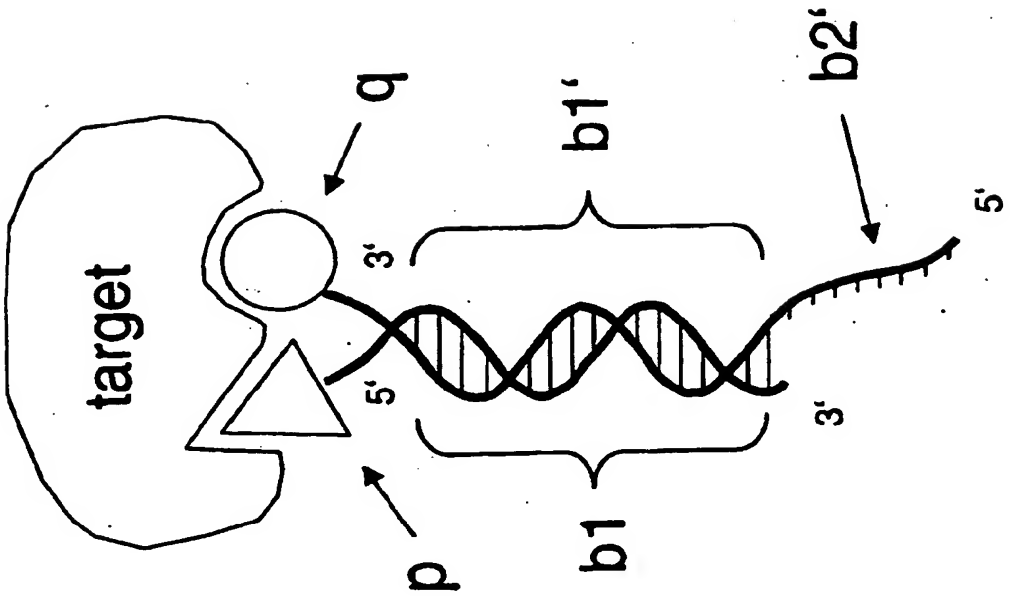


Fig. 10

REFERENCES CITED IN THE DESCRIPTION

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